

DRUG TREATMENT FOR
MALIGNANT HYPERPYREXIA

by

KEVIN LYONS AUSTIN

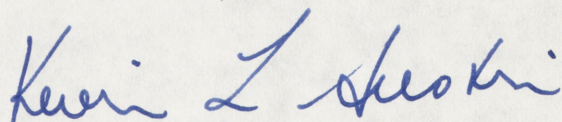
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STATEMENT

The results described in this thesis were obtained by myself under the supervision of Doctor Michael Denborough. Results obtained in collaboration with others are specifically acknowledged. In particular, the use of P-STAT as the programing system to analyse the data was suggested by Mr. Bill Craig, Department of Clinical Science, Australian National University.

The studies in this thesis were carried out during my employment as a Research Assistant Grade II in the Department of Clinical Science, Canberra Hospital, John Curtin School of Medical Research, Australian National University.



KEVIN L. AUSTIN

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This work would not have been possible without the expert help of the many surgeons who skillfully performed the muscle biopsies. I would also like to thank the patients who took part in this investigation. Without the normal muscle

biopsies, amounting to over eighty specimens during this investigation, no advance in the treatment of malignant hyperpyrexia would have been possible.

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PUBLICATIONS

"Treatment for Malignant Hyperpyrexia : Procaine or
Procainamide?"

K. L. Austin and M. A. Denborough (1975)

Clinical and Experimental Pharmacology and Physiology

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SUMMARY

The suddenness of the onset of anaesthetic induced malignant hyperpyrexia in apparently healthy subjects, and the high mortality rate associated with it, represent one of the most dramatic clinical events in modern medicine. The aetiology of the syndrome appears to include the detailed molecular structure of skeletal muscle cell membranes and the mechanism of calcium release. Muscle from susceptible patients responds abnormally to a variety of drugs and physical stimuli in vitro. High calcium levels in the myoplasm provide a rational explanation for the clinical and biochemical changes which occur during an episode of malignant hyperpyrexia.

The study presented in this thesis was undertaken to find the most suitable drug treatment. Methods employed included in vitro pharmacologic models of malignant hyperpyrexia based on normal human muscle. Drugs suggested for its treatment were evaluated and compared by measuring muscle response to drug administration in vitro. Muscle response to the drugs not only suggests a new drug treatment regime, but also specific biochemical changes in muscle membranes. Models of malignant hyperpyrexia based on normal human muscle were used to test drug efficacy and were supported by an in vitro study using malignant hyperpyrexia muscle.

A possible lesion in the control of phospholipid

fluidity in malignant hyperpyrexia membranes has been discussed and supported by some evidence. The proposed mechanism provides a rational basis for effects by local anaesthetics, calcium induced calcium release, general anaesthetics and the molecular action of dantrolene.

No drug suggested for the treatment of malignant hyperpyrexia has proved reliable. The overwhelming efficacy of dantrolene in suppressing and reversing the abnormal contractures in muscle from susceptible patients, suggests dantrolene is the most suitable drug for the treatment of malignant hyperpyrexia. The safety of dantrolene should prompt authorities to approve parenteral administration. It would appear that dantrolene is the only drug tested so far, that could increase the appalling survival rate in malignant hyperpyrexia.

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REFERENCES

SECTION I - PRELIMINARY OBSERVATIONS

A: Introduction

Malignant hyperpyrexia (hyperthermia) was first reported by Denborough and Lovell in 1960 as a potentially lethal, autosomal dominant syndrome of unknown aetiology. The condition they described consisted of a fulminant crisis triggered by potent inhalational anaesthetic agents, the outstanding features of which were fever, tachycardia, tachypnoea and cyanosis. In the few intervening years much has been learned about this remarkable pharmacogenetic disturbance of skeletal muscle.

The clinical features of the syndrome gradually emerged from various case reports, and a comprehensive statistical review of eighty-nine cases was published in 1970 by Britt and Kalow. A typical case consisted of a previously healthy patient, most often a young male, who was undergoing minor surgery. The first indication of anything unusual was often an abnormal reaction to succinylcholine. Enhanced fasciculations, a failure to relax or generalized muscular rigidity made intubation technically difficult to accomplish. Often a second dose of succinylcholine was unfortunately given in an attempt to overcome the muscular rigidity.

The next manifestation of the syndrome was variable, and included tachycardia, tachypnoea, hypotension, cyanosis and muscular rigidity. Hyperpyrexia was usually only

discovered when the patient's condition was rapidly deteriorating. All reports emphasised the rapid progression of the condition and also the difficulty in lowering the body temperature, even after packing the patient in ice. The majority of cases were fatal, death usually occurring within a few hours of the onset of the syndrome. It is now known that all individuals who are susceptible to malignant hyperpyrexia have an underlying disease of muscle, which may be subclinical (Denborough, Ebeling, King and Zapf, 1970; Isaacs and Barlow, 1970; King, Denborough and Zapf, 1972).

The discovery of an experimental animal, the Landrace pig (Hall, Woolf, Bradley and Jolly, 1966; Harrison, Biebuyck, Terblanche, Dent, Hickman and Saunders, 1968), the Pietrain pig (see refs in Hall, Lucke and Lister, 1975) and the Poland China pig (Nelson, Jones, Venable and Kerr, 1972) which suffered from a syndrome under general anaesthesia that appeared clinically identical to malignant hyperpyrexia, greatly assisted in the documentation of biochemical changes.

Investigations on susceptible pigs have shown that biochemical changes precede the development of both muscular rigidity and fever. The first major change in blood chemistry is a massive increase in lactic acid and a concomitant drop in blood pH.

By 1974 evidence supporting a biochemical lesion in skeletal muscle was mounting. Anaesthetic-induced malignant hyperpyrexia appears to result from increased free calcium levels in the myoplasm of skeletal muscle cells (Kalow, Britt,

Terreau and Haist, 1970; Moulds and Denborough, 1972; Nelson et al., 1972; Britt, Kalow, Gordon, Humphrey and Rewcastle, 1973; Harrison, 1973a; Britt, 1974). The source of this calcium in many human subjects seems to be the sarcoplasmic reticulum. (International Symposium on Malignant Hyperthermia, 1973; Britt et al., 1973; Harrison, 1973b; Moulds et al., 1972). Calcium release can be induced in susceptible muscle in vitro by a pharmacologically heterogeneous group of agents and also physical stimuli (Moulds and Denborough, 1974a). These effects are dependent upon extracellular calcium levels (Moulds et al., 1974; Nelson, Bedell and Jones, 1975), and are influenced by temperature (Nelson et al., 1975; Nelson, Austin and Denborough, unpublished observations, 1975).

Although these recent reports have added to our knowledge of the underlying cellular abnormality in malignant hyperpyrexia, associated advances in the treatment of this syndrome have been conspicuously absent (Hall et al., 1975). Apart from clinical approaches to the control of hyperpyrexia and acidosis, no specific drug therapy has proved reliable. Once the syndrome is established, it is often irreversible.

Thirty-six percent (36%) of survivors of malignant hyperpyrexia have had no specific drug therapy (Britt et al., 1970). Reports of successful treatment of the syndrome by a specific drug should therefore be treated with caution. The British Medical Journal in 1968 and Harrison in 1971 pointed out the lack of rationality in drug treatments for malignant hyperpyrexia. Since 1971 and up until late 1975, there have been six therapeutic regimes which have been reported as

successful. However, the mortality rate is still in the region of seventy percent (Clarke and Ellis, 1975). The use of procaine in the treatment of malignant hyperpyrexia was widely accepted as the best drug up until 1974. Numerous reports on its failure to control the syndrome began to appear and in 1975 there was extensive confusion in the literature as to its suitability in treatment regimes (Hall, Lucke and Lister, 1975). The lack of rationality in drug treatments that Harrison spoke of in 1971 was still present in 1975.

Hyperpyrexia may recur with repeated anaesthetics in susceptible patients, but the condition has also been encountered in patients who have previously undergone anaesthesia without apparent difficulty (Britt, 1974). The suddenness of the onset of the syndrome in apparently healthy patients and the high mortality rate associated with it, represent one of the most catastrophic clinical events in modern medicine. The need for drug therapy is clear. Simple cooling and correction of the acidosis, although extremely important in any treatment regime, do not guarantee survival (Clarke et al., 1975). The success of a particular therapeutic regime is measured by its action on in vitro muscle models or in vivo malignant hyperpyrexia pig reactions. Specific therapy has had varying degrees of success in the treatment of malignant hyperpyrexia in the Pietrain pig. Measures such as the administration of sodium bicarbonate, hyperventilation, fluid replacement and surface cooling are important, but in the established swine syndrome, additional therapy is necessary to inhibit the explosion of muscle

metabolism (Hall et al., 1975).

Methodological differences in the conditions for in vitro muscle studies of malignant hyperpyrexia have already contributed to the confusion in this field. Reports that muscle from patients susceptible to malignant hyperpyrexia was sensitive to halothane in vitro (Ellis et al., 1972; Moulds and Denborough, 1972) were negated by other workers (Kalow et al., 1970). However, a simple difference in temperature of the muscle incubation chamber has now been shown to be the cause of the conflicting reports. It is widely accepted that if muscle from malignant hyperpyrexia susceptible patients is tested in vitro at 37°C, exposure to halothane will induce contracture.

The failure of different workers to use standardised in vitro muscle procedures and the use of many models for malignant hyperpyrexia based on muscle from rats, rabbits, frogs, humans and malignant hyperpyrexia susceptible pigs made a comparative evaluation of drug efficacy in treatment regimes impossible (Harrison, 1973a; Clarke et al., 1975; Moulds et al., 1974a).

The difficulty in obtaining malignant hyperpyrexia muscle from humans for in vitro drug tests necessitates the selection of a model to evaluate drug efficacy. The choice of the most suitable model is discussed in a later section.

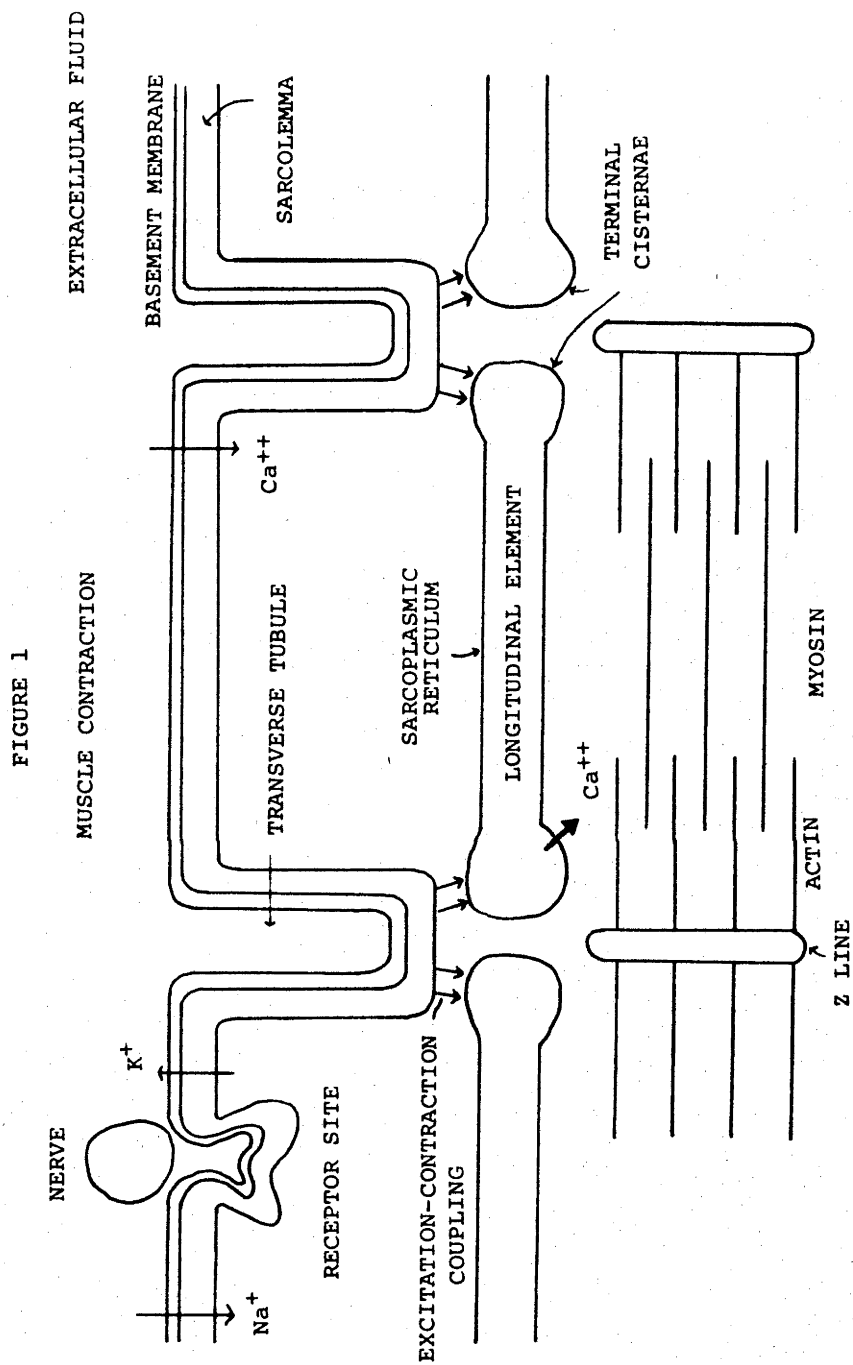
The general plea in the literature by a number of workers for an effective drug to reverse an already

established syndrome prompted the study presented in this thesis (Harrison, 1971; Hall and Lister, 1974). The study was designed to compare the relative efficacy of the major drugs suggested in antagonising calcium release from the sarcoplasmic reticulum. A model based on normal human skeletal muscle was selected, and the effects of procaine, procainamide, hydrocortisone, dexamethasone and dantrolene on isometric drug induced contracture were measured and compared. The relationship between the final release of calcium from sarcoplasmic reticulum and muscle contracture is well documented (Close, 1972; Britt, 1973; Cohen, 1975; Murray and Weber, 1974). The choice of the most suitable drug in treating malignant hyperpyrexia based on the normal model, was confirmed by testing drug efficacy in vitro on muscle from a patient susceptible to the syndrome.

The selection of the most suitable drug in the treatment of malignant hyperpyrexia does not imply that normal clinical procedures should be eliminated. At present the key to success in the management of acute crises is early cessation of all potent inhalational anaesthetics and all muscle relaxants. A comprehensive clinical treatment course is fully described by Britt (1974) and involves the use of sodium bicarbonate, furosemide, mannitol, glucose and insulin or potassium chloride, renal dialysis and external cooling or extracorporeal cooling if an expert cardio-vascular team is available.

Unfortunately such treatments are not sufficient to guarantee survival. The main aim of the present study was to

find the most effective drug therapy in the hope of increasing the appalling survival rate in malignant hyperpyrexia.



A diagrammatic representation of the sarcolemma, sarcoplasmic reticulum and the myofibrils during muscle contraction in skeletal muscle.

B: Normal skeletal muscle

i) Role of calcium

The skeletal muscle cell is enclosed by an outer basement membrane and an inner sarcolemma (see figure 1). At intervals a nerve ending is folded inward to the interior of the muscle fibre. These involutions greatly increase the area of nerve and muscle surfaces in contact with each other. Compared to the extracellular fluid the level of calcium within the myoplasm is low. During relaxation the sarcolemma can serve as a storage compartment for calcium ions and they are actively extruded from the myoplasm to the extracellular fluid across the surface membrane. At periodic intervals the sarcolemma is invaginated to form structures called transverse tubules (figure 1).

The sarcoplasmic reticulum appears to be the main site of calcium storage. It is composed of longitudinal elements, lying parallel to the muscle fibre axis and ends in enlarged terminal cisternae. These sacs are separated from the transverse tubules by partially bridged gaps. Usually each transverse tubule is associated with two terminal cisternae (figure 1). Lying below the transverse tubule are the Z lines to which contractile proteins called actin are attached at right angles. Thick filaments, composed almost entirely of myosin, are long rod-shaped molecules with two globular heads, and they lie parallel and in between the actin filaments. Troponin and tropomyosin make up the remaining two major proteins found in the contractile apparatus. A nerve impulse terminates at the

sarcolemma membrane. The impulse flows down the sarcolemma membrane into the transverse tubule, and the excited state of the transverse tubule is then transferred to the sarcoplasmic reticulum. Calcium ions are thought to be important in this transfer process. The calcium ions released from the transverse tubule induce a large release of calcium from the sarcoplasmic reticulum. This calcium has several effects. Muscle metabolism in general is stimulated, via activation of the phosphorylase system of enzymes, and thus the rate of glycolysis is increased. The calcium signal is detected and acted on by troponin and tropomyosin, which are positioned along the actin strand and with it constitute the thin filament. The calcium ions bind to the troponin, which then modifies the position of the tropomyosin molecules so that the myosin heads can contact the actin molecules. The contact of these two components results in shortening of a muscle fibril and thus muscle contraction.

The process whereby the 'electrically' excited sarcolemma is coupled to calcium release from the sarcoplasmic reticulum is known as the excitation-contraction coupling mechanism.

Calcium, in vivo, is released from the sarcoplasmic reticulum following depolarisation of the sarcolemma membrane. An electrical stimulus applied to muscle in vitro causing depolarisation of the sarcolemma, results in muscle contraction. A variety of drugs can also induce contracture, with or without depolarisation of the sarcolemma membrane.

Thus potassium chloride if added to the buffer surrounding a piece of skeletal muscle in a high enough concentration can cause artificial depolarisation, and contraction occurs. Caffeine if added at an appropriate level may also induce contracture without depolarisation of the sarcolemma.

The intracellular myoplasmic calcium concentration is modulated by three calcium pump sites to either sequester calcium intracellularly or to regulate calcium efflux from the cell. The sarcoplasmic reticulum and the mitochondria are capable of utilizing ATP to sequester calcium, while the calcium pump located in the surface membrane appears to be able to use the sodium gradient that exists across the membrane to transport calcium from the cell.

ii) Effect of caffeine, halothane and local anaesthetics

Although it has long been known that caffeine is able to produce a contracture of skeletal muscle (Ransom, 1911; Hartree and Hill, 1924) the first direct evidence to link the effect of caffeine with calcium movements within the muscle was published by Bianchi in 1961. He showed that caffeine increased both the influx and efflux of Ca^{45} from frog skeletal muscle. This effect was present even if the fibres were depolarised with high external potassium concentrations.

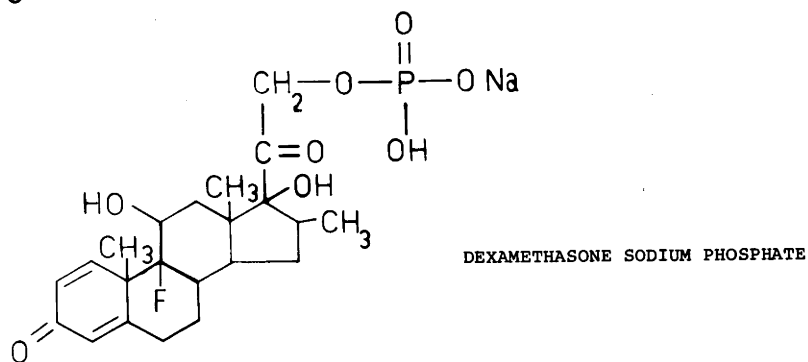
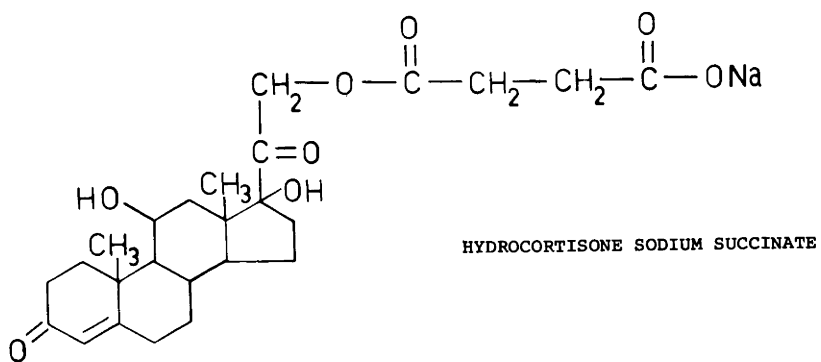
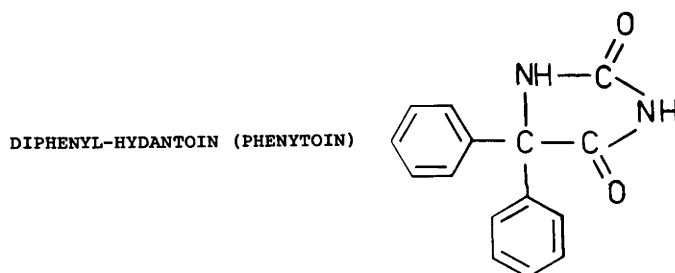
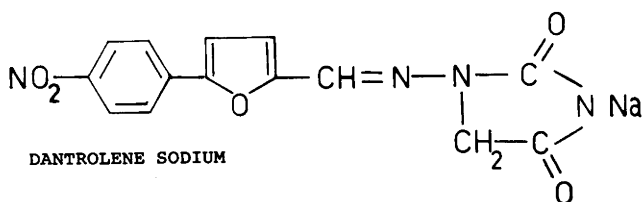
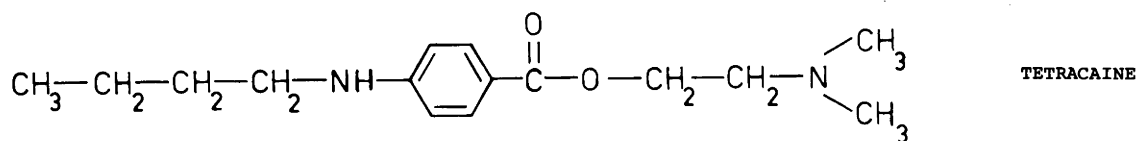
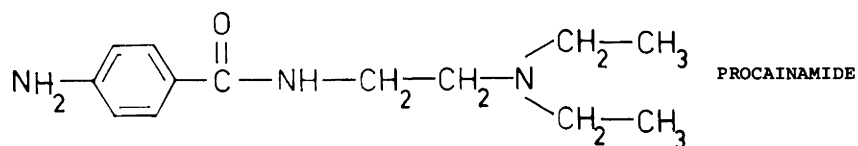
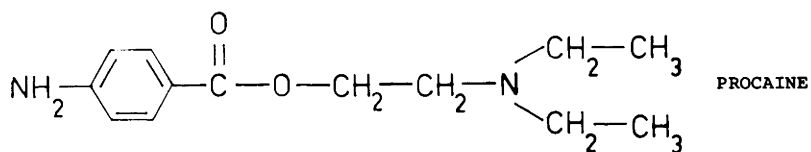
Evidence accumulated over the 1960's and early 1970's on the site of action of caffeine in skeletal muscle. Caffeine appears to have three distinct actions on frog sartorius muscle in relation to contracture. At low

concentrations (less than 1 mM) caffeine acts primarily on sites related to the coupling of the action potential to muscle contracture (Sandow and Preiser, 1964; Sandow, Taylor and Preiser, 1965; Brust, 1965; Sandow and Brust, 1966; Bianchi et al., 1967). Intermediate concentrations of caffeine (1-5 mM) cause a contracture which does not require membrane depolarisation and is reversible upon removal of caffeine (Axelsson and Thesleff, 1958; Matsushima, Fujino and Nagai, 1962). Concentrations of caffeine higher than 5 mM cause rigor or irreversible contracture. The site of action of caffeine is believed to be the sarcoplasmic reticulum where it can cause a release of calcium and also block the uptake of calcium thus maintaining a high calcium level in the myoplasm (Herz and Weber, 1965; Weber and Herz, 1968).

Strobel and Bianchi (1971) demonstrated that halothane potentiated the caffeine contracture and this potentiation was prevented by procainamide. Feinstein (1963) had previously shown that procaine inhibited caffeine contractures and caffeine induce rigor.

An important finding was the first documentation by Kalow et al. (1970) that halothane potentiated the contracture produced by caffeine in human skeletal muscle. This was confirmed by Moulds and Denborough in 1972, who also demonstrated that the potentiation was inhibited by procaine. A comprehensive study on the effects of caffeine, halothane and procaine on normal human muscle was published in 1974 by Moulds and Denborough (1974a). Normal human

FIGURE 2



skeletal muscle is more sensitive to caffeine and halothane than frog sartorius muscle, with 4 mM caffeine producing a slow small contracture in human muscle but producing rigor in frog muscle. Halothane does not usually induce a contracture in normal human muscle in vitro but small contractures may occur (Moulds and Denborough, 1974a; Nelson, Austin and Denborough, paper in preparation).

Although the detailed molecular actions of halothane and caffeine are not known, the evidence available suggests that the excitation-contraction coupling mechanism is involved and that halothane does not inhibit the uptake of calcium into the sarcoplasmic reticulum (Moulds et al., 1974b).

The local anaesthetics procaine and tetracaine (see figure 2) block caffeine and halothane potentiated caffeine contractures in human muscle as well as muscle from a variety of animals (Austin and Denborough, 1975; Bianchi and Bolton, 1967). Some local anaesthetics can actually augment caffeine contractures however, and a detailed mechanism involving the charge state of the anaesthetic is reviewed by Seeman (1972).

C: Malignant hyperpyrexia skeletal muscle

i) Role of calcium

The detailed molecular mechanism in malignant hyperpyrexia involving intra-cellular muscle membranes and calcium movements cannot be fully realized until excitation-contraction coupling in normal muscle is more clearly

understood.

With respect to treatment, however, a detailed molecular view may not need to be established. The exact site of the primary lesion in malignant hyperpyrexia, if only a single lesion exists, may not be critical to studies on drug treatment. As long as the drugs studied inhibit calcium release from the sarcoplasmic reticulum, control of abnormal muscle response may be possible. The membrane abnormality may be present in the sarcolemma, in the sarcoplasmic reticulum, in the association of these two membranes or in mitochondria. At present no distinction between any of these possibilities can be made.

The general control mechanism of calcium movement in skeletal muscle from malignant hyperpyrexia patients appears to be normal and any abnormality only becomes evident upon exposure to halothane or succinylcholine. Most of the in vitro work on whole muscle fibres and isolated membrane preparations has therefore been focussed on the effects of the clinical triggering agents.

ii) Effect of caffeine, halothane and local anaesthetics

Kalow et al. (1970) reported that muscle from patients susceptible to malignant hyperpyrexia gave an enhanced caffeine contracture and that halothane potentiated the caffeine response to a greater degree than that found in normal muscle. Although these workers stated that treatment with halothane alone did not cause a contracture, the temperature of the muscle buffer was 25°C. It has since

been shown that if the physiological temperature of 37°C is selected, a spontaneous contracture occurs upon exposure to halothane (Ellis et al., 1972; Moulds et al., 1972).

Recently, Moulds and Denborough (1974b) have shown that muscle from susceptible patients is supersensitive to potassium chloride, succinylcholine, caffeine, halothane and caffeine potentiated halothane treatments. They also reported that procaine partially reversed and inhibited halothane and succinylcholine contractures. This effect of procaine on halothane contractures in vitro has also been reported by Ellis, Keaney and Harriman (1973), Harriman, Sumner and Ellis (1973) and Keaney and Ellis (1971).

Concentrations of caffeine too low to elicit a response in normal muscle (less than 4 mM) produce a large sharp response in malignant hyperpyrexia muscle (Moulds et al., 1974b). Since it is thought that low levels of caffeine affect the excitation-contraction coupling mechanism in skeletal muscle, sensitivity to low caffeine levels in effected muscle may reflect an excitation-contraction coupling abnormality. The speed of relaxation following such a contracture suggests that caffeine has no effect on the re-uptake of calcium into the sarcoplasmic reticulum (Moulds et al., 1974b). Contractures of normal muscle induced by 8 mM caffeine have a slow relaxation rate following peak tension, suggesting that this level of caffeine affects the calcium ATPase of the sarcoplasmic reticulum (Moulds and Denborough, 1974b).

D: Treatment of malignant hyperpyrexia

i) Suggested drug treatment regimes and their rationale

The use of procaine and procainamide (see figure 2) was based originally on the finding by Feinstein in 1963, that procaine blocked the rigor produced in frog muscle by caffeine.

Others later found that procaine would block or reverse contractures in malignant hyperpyrexia muscle induced by halothane in vitro (Moulds et al., 1972). For theoretical reasons procaine, which is known to accelerate calcium uptake into the sarcoplasmic reticulum and block its release, should be more effective in malignant hyperpyrexia than lignocaine which enhances the release of calcium from the same membrane.

In a preliminary report it was shown that procaine and procainamide inhibited and relaxed caffeine and caffeine + halothane contractures in normal human muscle to differing degrees (Austin and Denborough, 1975). The treatment rationale may be summarised: malignant hyperpyrexia appears to result from an increased level of calcium in the myoplasm; the source of this calcium appears to be the sarcoplasmic reticulum; procaine and procainamide have both been shown to reduce the release of calcium from the sarcoplasmic reticulum and they should therefore be useful in controlling the syndrome in humans.

In contrast to the rationale for the use of procaine and procainamide, the use of dexamethasone and hydrocortisone

(see figure 2) appears to be empirical.

Dexamethasone and hydrocortisone effects on muscle metabolism have not been documented. The effects of these two drugs on normal muscle contractures in vitro induced by caffeine and caffeine + halothane are not known. A single report on the inhibitory effect of hydrocortisone on halothane induced contractures from a susceptible patient was made by Ellis et al. (1974). The in vitro test was supposed to support the apparently successful use of dexamethasone in treating a nitrous-oxide induced case of malignant hyperpyrexia. No reason was given as to why dexamethasone was chosen for the primary drug therapy. On the basis of this report treatment regimes including dexamethasone as the primary drug choice have already been tried (Raitt and Merrifield, 1974). An evaluation of the efficacy of these two steroids in antagonising in vitro muscle contracture was obviously required. Both dexamethasone and hydrocortisone were included in the present study.

Reports that dantrolene (see figure 2) could block excitation-contraction coupling without affecting caffeine contractures (Putney and Bianchi, 1974), neuromuscular transmission or electrical properties of muscle (Ellis and Carpenter, 1972; Ellis and Bryant, 1972; Ellis, Castellion, Honkomp, Wessels, Carpenter and Halliday, 1973) prompted Nelson and Denborough to use this drug to investigate its efficacy in antagonising contractures in normal human muscle (Nelson and Denborough, 1976, paper in preparation). The potency of its muscle relaxation properties is well documented

(Ellis et al., 1973), and prompted Harrison into using the drug to control malignant hyperpyrexia in swine. Harrison (1975) demonstrated that dantrolene could be used to block initiation of malignant hyperpyrexia by halothane and also to terminate the established syndrome in swine. He reported a 100% survival rate when a high enough concentration of dantrolene was selected. Its apparent ability to interfere with the excitation-contraction coupling mechanism and caffeine-induced contractures in normal human muscle warranted further investigation.

ii) Selection of in vitro drug concentrations

Selection of appropriate in vitro drug concentrations is complex. Even when blood concentrations of a particular drug are well documented, the blood levels do not necessarily correlate with tissue levels. Doses recommended by a manufacturer and doses used by clinicians do not always agree and with some drugs there are wide discrepancies. Toxic levels of drugs may vary from one patient to another. Reports on successful treatment of malignant hyperpyrexia include a wide range of concentration units. Dosage levels reported in mg/Kg, total mg, mls of x%, mM final concentration, $\mu\text{g/ml}$ as well as other units add a great deal of confusion. Drug levels tolerated by animals may produce toxic effects in humans.

As the main purpose of this study was the selection of the most appropriate drug for treatment of malignant hyperpyrexia, great care was required in the selection of in vitro concentrations. Indeed, inappropriate selection of

in vitro drug concentrations by a number of workers, has led to recent criticism (Hall and Lister, 1974).

The use of procaine in the treatment of malignant hyperpyrexia has been suggested at levels many times the accepted toxic dose. The concentration of procaine used in model studies in vitro with caffeine-induced muscle contracture has varied from 2 mM to 10 mM (Beldavs, Small, Cooper and Britt, 1971; Relton, Steward, Creighton and Britt, 1972; Hall et al., 1972; Kalow et al., 1970; Britt et al., 1973; Feinstein, 1963; Bianchi and Bolton, 1967). Halothane-induced contracture in muscle from susceptible patients has been shown to be reduced by 1.8 mM to 5 mM procaine (Moulds and Denborough, 1972; Ellis, Keaney and Harriman, 1973; Hall and Lister, 1974).

These concentrations are higher than those recommended for clinical use. Not only would procaine concentrations of this magnitude induce hypotension but the rapid hydrolysis of procaine in the blood suggests the levels might be difficult to maintain (Wikinski et al., 1970; Hall and Lister, 1974).

Recommended clinical doses of dexamethasone to adults, range from 2 mg to 40 mg as a single intravenous injection. Further injections every 2 to 6 hours may be required for the treatment of severe shock (Oaks and Cohen, 1967; Cavanagh and Singh, 1970). Rare dosages almost ten times those just mentioned have been reported making the selection of a clinical concentration difficult (Dietzman,

Ersek, Bloch and Lillehei, 1969).

Two concentrations of each drug were selected to make allowances for such discrepancies, and to provide information about drug efficacy in relation to dosage. The lower concentration was selected to fall in the usual clinical range and the higher concentration of each drug studied, may be reached in emergencies. The difference, if any, between the two concentrations of a particular drug, would help to decide if the increased concentration, with the concomitant increased danger, was necessary or worthwhile.

Dantrolene Sodium

In contrast to the numerous reports on the effectiveness of dantrolene in controlling a variety of muscle complaints in humans, little is known about the blood concentrations that can be tolerated. Although dantrolene was first noted to have muscle relaxant properties in 1967, oral administration to humans has only been sanctioned in the United Kingdom since February, 1975. Parenteral use has yet to be approved in the United Kingdom or the United States of America (Snyder, Davis, Bickerton and Halliday, 1967; Ellis et al., 1975). Dantrolene is not yet available in Australia.

Clinical evaluation of the safety of dantrolene is therefore based on oral dosages. A typical treatment regime for disorders of the central nervous system would be 100 mg dantrolene by tablet four times a day. With this dose, blood levels of 2-3 $\mu\text{g/ml}$ (6-9 μM) are attained with peak concentrations as high as 3.75 $\mu\text{g/ml}$ (11 μM) (Chyatte and

Birdsong, 1971; Chyatte, Birdsong and Bergman, 1971; Herman, Mayer and Newcombe, 1972). A single dose of 150 mg orally results in a blood level in a normal adult of about 1.3 $\mu\text{g/ml}$ (4 μM) four hours after ingestion (Herman et al., 1972). Assuming a blood volume of 5 litres and 100% absorption of the drug into the blood, an initial 150 mg dose would lead to a concentration of 30 $\mu\text{g/ml}$ (89 μM). However, only about 4% of this theoretical concentration is achieved. Failure of absorption from the intestine, hydrolysis or chemical degradation in the blood, deposition into tissues and excretion all play important roles in controlling steady-state blood levels of any drug. Because little is known about the concentration of drugs in tissues, blood levels are generally used in in vitro experiments.

Harrison (1975) has suggested that dantrolene blood concentrations of about 80-100 $\mu\text{g/ml}$ (237-297 μM) are necessary to prevent the onset of porcine malignant hyperpyrexia. Concentrations of dantrolene of this magnitude have not been achieved in humans, and a rising list of some minor side effects during clinical trials suggests that concentrations of dantrolene about 6-7 $\mu\text{g/ml}$ (18-21 μM) should not be exceeded (Chyatte and Birdsong, 1971).

The two concentrations of dantrolene selected for use during this study were 6 μM and 30 μM ; a level of 6 μM being easy to attain clinically and a level of 30 μM , which might be warranted in an emergency.

Procaine Hydrochloride

Procaine is rapidly hydrolysed in human blood (Wikinski, Usubiaga, and Wikinski, 1970; Hall and Lister, 1974). Since the rate of hydrolysis probably varies and tissue levels are difficult to correlate with blood levels, the steady-state blood levels of procaine are practically unknown. Although toxic levels in blood are known for laboratory animals such as dogs, very little is known about toxic levels in humans (Wikinski et al., 1970). A dose of 6.8 g is required, in theory, to produce a concentration of 5 mM in the circulation of an adult (Moulds and Denborough, 1972). However, the effect of procaine on caffeine-induced muscle contracture is dose dependent and so levels lower than 5 mM may still be useful (Moulds and Denborough, 1972).

Harrison (1971), on the basis of procaine treatment with malignant hyperpyrexia susceptible pigs suggested a loading dose of 30-40 mg/Kg (1.5-2.1 mM) in humans, but this concentration will almost certainly lead to convulsions and circulatory failure (Usubiaga et al., 1966; Wikinski et al., 1970; Hall and Lister, 1974). Hall and Lister (1974) suggest that 2 mM blood levels of procaine cannot be obtained safely while Usubiaga (1966) and his associates regard 20-80 µg/ml (0.07-0.3 mM) as toxic in humans.

Attempts to achieve a dosage of 30 mg/Kg (~1.5 mM) appear to have contributed to the death of a patient in 1974 (MacLachlan and Forrest, 1974). Severe hypotension was induced by procaine after only 600 mg of the suggested dose of 2,000 mg had been given over twenty minutes (Harrison, 1971).

The hypotension was not reversed by isoprenaline. Rapid loss of active procaine levels in blood from 800 to 96 µg/ml occurs within five minutes of infusion (Wikinski et al., 1970). A loading dose of 600 mg over twenty minutes would probably result therefore in a relatively low blood level of procaine.

Although the patient described by Wikinski and his associates (1970) survived a procaine dosage of 4,000 mg, they ascribed the survival to youth, hyperventilation with 100% oxygen, his otherwise good health and a lot of luck. Hyperventilation with 100% oxygen is known to inhibit and help reverse cardiac depression caused by overdoses of intravenous local anaesthetics (Doas, Lopez and Virtue, 1963). Wikinski's patient was being hyperventilated with 100% oxygen as the accidental overdose occurred.

In summary reasonably safe intravenous procaine administration probably ranges from 200-700 mg (0.1 -0.5 mM). The levels of procaine selected for this study were 0.5 mM as a concentration which was relatively safe and 5 mM.

Procainamide Hydrochloride (Pronestyl)

The clinical use of procainamide is similar to that for procaine. They are often used intravenously as anti-arrhythmic agents. Safe blood levels seem to correlate with levels possible with procaine. Dosages as high as 700-900 mg (0.5 mM - 0.7 mM) and as low as 200 mg (0.1 mM) have been used in the successful treatment of malignant hyperpyrexia (Hall and Lister, 1974; Noble, McKee and Gates, 1973; Brebner and Jozefowicz, 1974).

The low concentration of procainamide used in this study (0.5 mM, 679 mg) was selected to fall in the middle of the range mentioned above and the high concentration was 5 mM.

Dexamethasone and hydrocortisone

The use of dexamethasone or hydrocortisone in treatment regimes is relatively recent (Ellis et al., 1974; Raitt and Merrifield, 1974; Clarke and Ellis, 1974). Apart from the three references just cited no other serious attempt to control malignant hyperpyrexia by dexamethasone or hydrocortisone has occurred, to the knowledge of this author. Inadvertant use of steroids has occurred during treatments, but in these instances, relatively low levels have been used (Noble et al., 1973).

Two successful treatment courses have been reported with dexamethasone (Ellis et al., 1974; Raitt and Merrifield, 1974). An 11 year old girl received 45 mg and the treatment initiated by Raitt was 4 mg (0.2 mg/Kg) to a 6 year old boy. Ellis et al. (1974) suggests dexamethasone doses of 1-2 mg/Kg (27-54 μ M). Clarke and Ellis (1974) feel that 100 mg (39 μ M) dexamethasone as well as procaine should be given to an adult who develops malignant hyperpyrexia. Interestingly, the use of dexamethasone was suggested because of the peripheral vasodilation and positive inotropic effects on the myocardium, not because of any direct effect on skeletal muscle metabolism.

In the same publication that Ellis and his colleagues reported the successful treatment of malignant hyperpyrexia with dexamethasone, they presented in vitro evidence for an effect by hydrocortisone.

Although these results were reported as preliminary, no conclusion can be drawn on the efficacy of hydrocortisone at clinical concentrations in inhibiting halothane-induced contractures. Results from the same study, do provide evidence however, that clinical levels of hydrocortisone (29 μM) can promote relaxation of a halothane-induced contracture. The successful use of steroids in the treatment of malignant hyperpyrexia reported by a number of workers and its inadvertant use by a few others during successful treatments, warrants a more detailed investigation.

A normal clinical dose of dexamethasone in the treatment of a large variety of disorders ranges from 2 mg to 20 mg (0.8 - 8 μM). Doses as high as 40 mg (16 μM) are used occasionally. However, a few instances of dexamethasone therapy about ten times this figure (~400 mg, 160 μM) have been reported (Dietzman et al., 1969). Similar variations in hydrocortisone administration are also seen with clinical doses via intravenous injections ranging from 100 mg to 1000 mg (55 - 552 μM).

The low concentrations of dexamethasone and hydrocortisone used during this study were equivalent to dosages of 20 mg (7.8 μM) and 100 mg (55 μM) respectively. These levels are easily obtained clinically with very few

side effects. The higher concentration of dexamethasone was 200 mg (77.5 μ M) and 1000 mg (552 μ M) for hydrocortisone.

SECTION II - SELECTION OF THE MOST EFFECTIVE DRUG IN
THE TREATMENT OF MALIGNANT HYPERPYREXIA

A: Introduction

Anaesthetic-induced malignant hyperpyrexia appears to result from increased free calcium levels in the myoplasm of skeletal muscle cells (Kalow et al., 1970; Moulds and Denborough, 1972). Raised myoplasmic levels of calcium can be induced in susceptible muscle in vitro by a variety of drugs and physical stimuli (Moulds and Denborough, 1974b). Malignant hyperpyrexia muscle is more sensitive than normal muscle to these same drugs and physical stimuli. The exact site of the lesion in malignant hyperpyrexia is unknown, but the sarcolemma, sarcoplasmic reticulum and mitochondria have received most of the attention in recent years.

The lack of advancement in the treatment of malignant hyperpyrexia can be attributed primarily to the shortage of material. Malignant hyperpyrexia is a rare syndrome and muscle becomes available for testing only occasionally. Various models for malignant hyperpyrexia have therefore been proposed in an attempt to overcome this problem. The in vitro models include the use of normal human, frog and rat muscle, as well as pig muscle from susceptible swine.

B: Selection of an appropriate in vitro model for
malignant hyperpyrexia

Models of human malignant hyperpyrexia based on normal muscle from pigs, rabbits, rats or frogs may not be

relevant, because of inter-species differences in muscle sensitivity to caffeine and halothane in vitro (Nelson, Bedell and Jones, 1975; Clarke and Ellis, 1975; Feinstein, 1963; Austin, Nelson and Denborough, unpublished observations). Although porcine malignant hyperpyrexia has been suggested as a suitable model of the human syndrome, workers have recently thrown doubt on its suitability (Britt, Endrenyi and Cadman, 1975). Even though basic differences between the two syndromes have been documented (Britt et al., 1975) many similarities warrant its further use as a model for malignant hyperpyrexia.

Drug treatment, however, implies human drug response. A particular drug effect in pig, rabbit or rat muscle does not necessarily guarantee the same effect in human muscle. The selection of a single model for a comparative study of the many drugs now suggested as suitable treatments for malignant hyperpyrexia was required.

Recent studies on the action of various pharmacologic agents on human muscle by Moulds and Denborough (1974a) have enabled a suitable human model to be proposed. Muscle from susceptible patients develops a greater than normal in vitro contracture in the presence of caffeine (Kalow et al., 1970), halothane, potassium chloride and succinylcholine (Moulds and Denborough, 1974b). Caffeine contractures are potentiated to a greater than normal extent by the addition of halothane (Britt et al., 1973; Ellis and Harriman, 1973).

Treatment of normal muscle with low concentrations of caffeine (2 mM) does not result in contracture. However, pretreatment of normal muscle with low concentration of caffeine (2 mM) potentiates a response to halothane (Moulds and Denborough, 1974a). Caffeine at levels of 8 mM induces contracture of normal human muscle in vitro (Moulds et al., 1974a; Nelson, Austin and Denborough, unpublished observations). Caffeine-induced contractures and caffeine potentiated halothane-induced contractures in normal human muscle have both been suggested as suitable models for the in vitro study of malignant hyperpyrexia (Moulds et al., 1972; Kalow et al., 1970; Britt et al., 1973).

The description of a study based on these two models for malignant hyperpyrexia follows. Two basic types of experiment were performed. A drug added before the contracture-inducing agents was an in vitro model to test drug prevention of malignant hyperpyrexia, while a drug added during a contracture was intended as a model to test drug reversal of the established syndrome.

The following experiments were designed to measure and compare the efficacy of six drugs in inhibiting and relaxing caffeine and caffeine potentiated halothane muscle contractures in vitro.

C: Materials and methods

i) Preparation of solutions

The buffer used to incubate the human muscle during this study was basically as described by Elford in 1970.

Muscle buffer which was continuously bubbled with carbogen (95% oxygen/5% carbondioxide) contained the following as final concentrations at pH 7.3: NaCl 121.2 mM, KCl 5.4 mM, CaCl_2 2.5 mM, MgSO_4 1.2 mM, NaH_2PO_4 1.2 mM, NaHCO_3 15.0 mM and glucose 11.5 mM. The muscle buffer was prepared daily from concentrated stocks by appropriate dilution to prevent contamination by fungal growth which occurred if the buffer was kept more than 72 hours.

Caffeine was freshly prepared before each experiment at a stock concentration of 100 mM in the standard muscle buffer.

It was technically difficult to administer both sudden and yet constant levels of halothane to the muscle buffer because of the volatility of this agent at 37°C. A halothane vaporiser (DRÄGER VAPOR) was therefore selected which delivered a constant level of halothane to a stream of carbogen, which was then directed to the muscle bathing chamber. The VAPOR was calibrated and capable of delivering any level of halothane between 0.05% and 3.5%. Variations in the concentration of halothane with changes in room temperature were automatically compensated for by changing the instrument setting. The working range of the VAPOR was between room temperatures of 16°C and 30°C. Neither gas pressure nor gas flow affected the accuracy of the VAPOR.

Dantrolene sodium was prepared daily, as a stock solution of 148.7 μM in 10 mM NaOH. Initial attempts to prepare solutions of dantrolene in the standard muscle buffer

resulted in a milky-white suspension. An equal volume of buffer was removed if the volume of any drug to be added exceeded 1 ml. The resultant 20% change in the ionic composition when 5 mls of dantrolene was added did not appear to affect muscle response. Control experiments were performed to confirm that 2 mM NaOH (final concentration) did not affect muscle response.

Dexamethasone was supplied by the hospital pharmacy as dexamethasone sodium phosphate dissolved in water (8.5 mM). Dexamethasone sodium phosphate is a water soluble inorganic ester of Decasone and is about 25 to 30 times more potent than hydrocortisone. Sodium bisulfite (0.1%), methylparaben (0.15%) and propylparaben (0.02%) were added to the Decasone as preservatives by the manufacturer.

Hydrocortisone was supplied as Efcortelan Soluble. Efcortelan is a soluble ester of hydrocortisone and was supplied as a freeze-dried powder which was dissolved in water immediately before addition to the muscle bathing chamber. The standard vial contained 100 mg of hydrocortisone as hydrocortisone sodium succinate in 2 mls of water (137.9 mM), buffered with Na_2HPO_4 and NaH_2PO_4 .

Pronestyl was supplied as a 10% sterile aqueous solution (367.6 mM). Pronestyl for intravenous use contains benzyl alcohol (0.9%) and sodium bisulfite (0.09%) as preservatives. It is the amide analogue of procaine hydrochloride and is synonymous with procainamide. The pH had been adjusted to between four and six with HCl or NaOH by

the manufacturer. Such small volumes of pronestyl were being added to the muscle bath that no change in the pH of the buffer occurred.

Procaine and procainamide were both prepared as stock solutions of 250 mM in the standard muscle buffer at pH 7.3.

Dantrolene was a gift from the Norwich Pharmacal Company, U.S.A., and halothane was purchased from I.C.I. Australia. Procaine and procainamide were supplied by the Sigma Chemical Company and K & K Laboratories of the U.S.A. respectively. Pronestyl was manufactured by E. R. Squibb and Sons Pty. Ltd., Vic.; Efcortelan Injection by Glaxo Australia Pty. Ltd., Vic.; Decasone by Charles E. Frosst Australia Pty. Ltd., and caffeine by the Sigma Chemical Company, U.S.A.

ii) The biopsy technique

The biopsy technique was based on the method developed by Moulds and Denborough (1974a). Strips of rectus abdominis muscle were obtained from patients undergoing elective abdominal surgery.

It was found necessary to attend each operation so that quick incubation of the muscle specimen was assured. Immediate immersion in buffer and aging of the muscle after biopsy were stressed by Moulds (1974) and were confirmed in this study to be of paramount importance. The biopsy was invariably performed at the start of each operation, before halothane was administered to the patient. Premedication agents and drugs used to intubate patients were found to have no effect on muscle response, confirming the finding by Moulds (1974).

Human muscle shortened upon removal from a patient. A 3 cm length of muscle would shorten considerably in vitro sometimes as much as 50% of the length in vivo. There was some evidence to suggest that if shortening occurred with pig muscle, then the tension developed by a fibre would be drastically lowered (Nelson, 1974, personal communication). An attempt to control shortening included the development and routine use of a simple muscle biopsy clamp (figure 3). The clamp also ensured a fixed length and depth of muscle would be routinely obtained.

The muscle clamp shown in figure 3 was sterilized and presented to the surgeon immediately before each

FIGURE 3

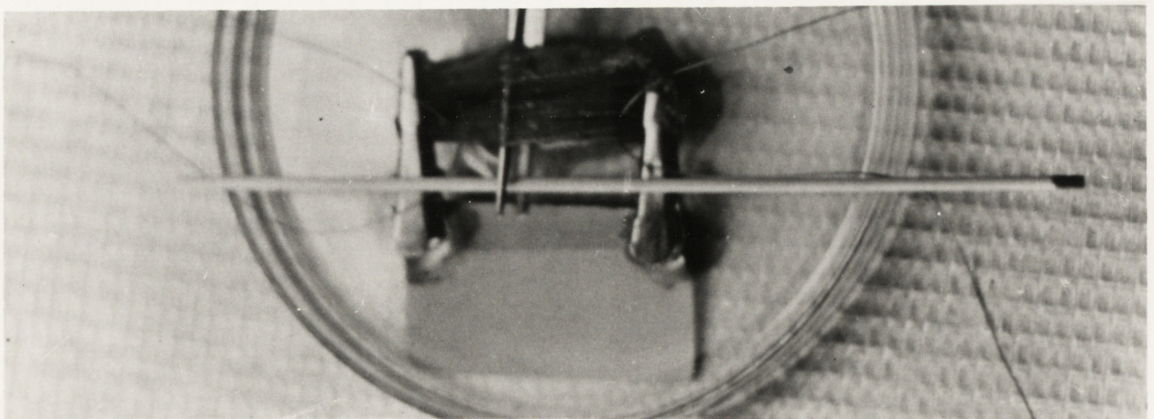
A MUSCLE BIOPSY CLAMP



$1\frac{1}{2}$ "

FIGURE 4

DISSECTION OF MUSCLE INTO FIBRE BUNDLES



operation. As soon as the surgeon made an incision through the rectus abdominis sheath, the clamp was placed over a section of the sheath at the site of incision. The clamp, with muscle attached between the two clips, was then removed from the patient and placed in standard muscle buffer at 37°C.

The muscle specimen was then transported to the laboratory and placed in a large petri dish containing more buffer. The first incision was through the thinnest face of the muscle. A pair of forceps was inserted into the incision (figure 4) in order to separate the two distinct 'planes' of muscle.

Bundles of fibres from the sample were isolated (2 cm x 2 mm x 2 mm) and small stainless metal rings tied on each end (figure 6). They were then placed in buffer at 37°C with carbogen bubbling through the buffer continuously. Care was taken to ensure that the rings were not tied on too tightly. If this occurred, the thread would usually cut the muscle and muscle specimens would frequently break when contracture was induced. The finer the thread used the more samples would break due simply to cut muscle. A thicker thread, Ethicon size '00', was therefore selected and routinely used.

During 1975 it was noted that inter-fibre variation and fibre breakages were reduced in experiments with pig muscle, by tying applicator sticks to muscle fibres before sectioning them from the pig (Nelson, Austin and Denborough,

FIGURE 5

A MUSCLE FIBRE BUNDLE TIED TO A STICK

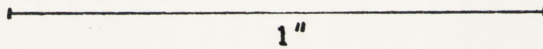
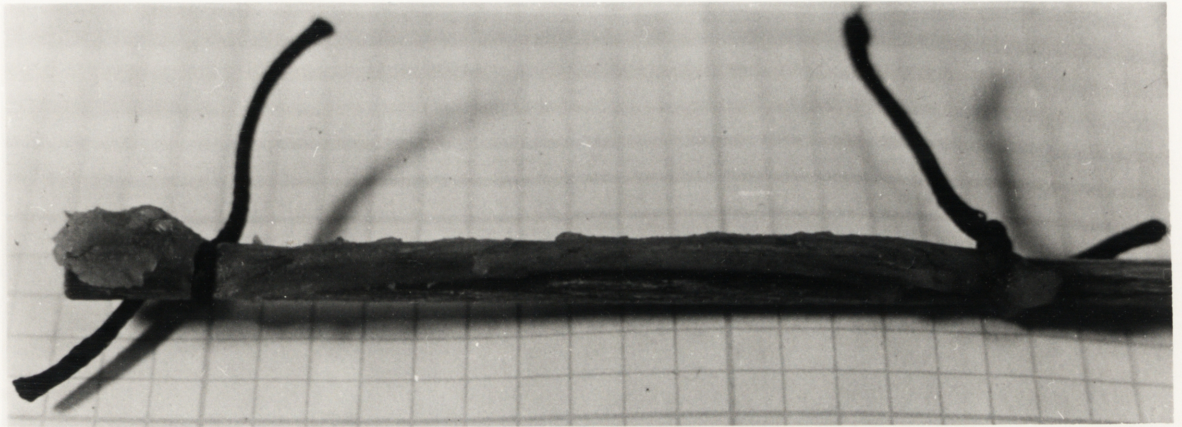
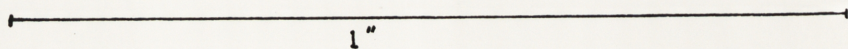


FIGURE 6

A MUSCLE FIBRE BUNDLE TIED TO TWO RINGS



unpublished observations). The technique ensured that muscle fibres did not shorten and that no stretching took place. The rings were tied on to the muscle just before the fibres were cut off the sticks, ready for testing. On closer examination of the human technique it was found that some fibres were breaking during contracture even though extreme care had been taken not to cut the muscle with the thread. It was also noted that more fibres broke towards the end of the day, perhaps up to 6 hours after removal from the patient. Fatigue and deterioration around the site of ring attachment was clearly evident when fibres were examined closely. The area affected was restricted to about 1 mm on either side of the thread. Deterioration also took place with pig muscle where it was tied onto the stick, but when rings were tied on just before use they were placed a short distance away from the site of attachment, thereby avoiding the weakened area.

The human technique during this study was therefore extended to avoid muscle breakages by using applicator sticks. Moulds (1974) had demonstrated that the muscle had to be tied very shortly after removal from a patient, and so sticks were used to facilitate this pre-requisite as well. Individual bundles of fibres were dissected carefully but not removed from the clamp. As each bundle was ready, an applicator stick was laid next to it, tied and then removed as shown in figure 5.

The fibres tied on to the sticks were then placed in buffer at 37°C and continuously bubbled with carbogen.

When a fibre bundle was required, the rings would be tied onto the fibre, the fibre cut from the stick and placed into the muscle bathing chamber. The improvements in contracture were threefold; increased absolute contracture tension; decreased fibre-fibre variation between fibres from the same patient and greatly decreased incidence of muscle breakages during contracture.

Improvements in absolute tension during this study account for the apparent wide range of control tensions in some cases but were continuously monitored by appropriate controls. During the final stages of this study all fibres were routinely tied to sticks.

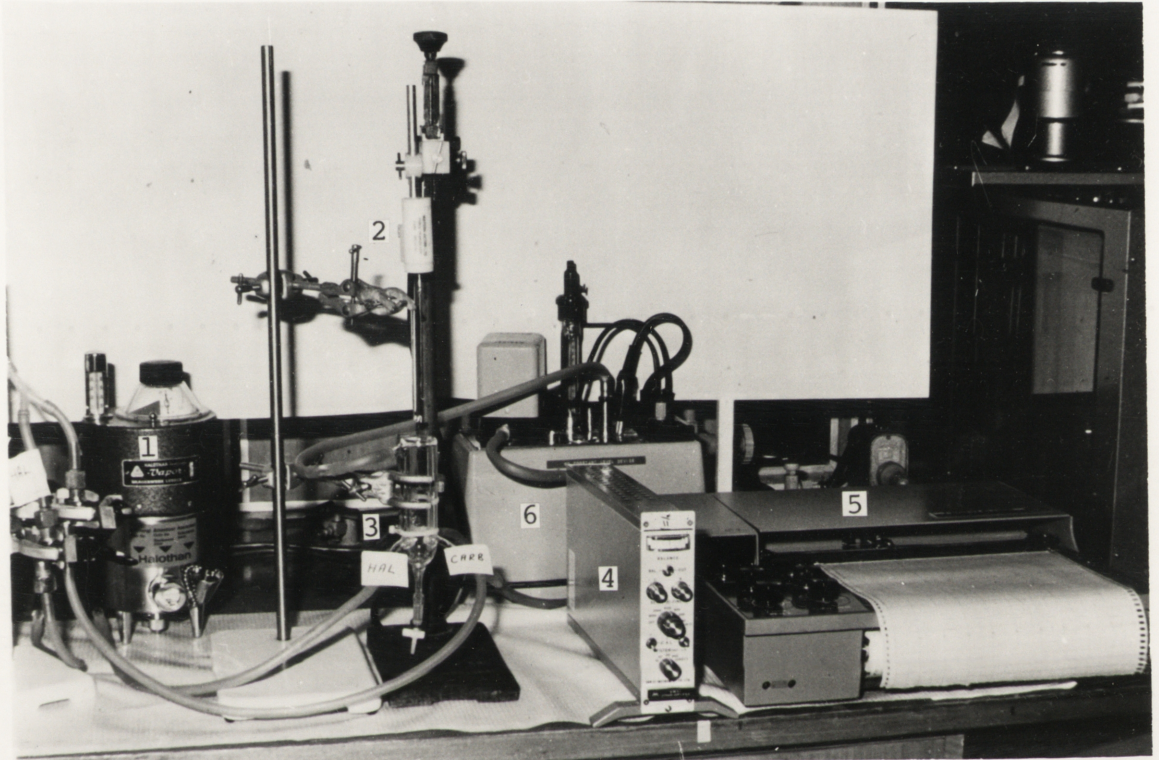
iii) Measurement of muscle response

For the test, a fibre bundle was either removed from the incubation vessel with the small metal rings attached, or removed from a stick with rings which had just been tied to it. The preparation was then added to a vertical 25 ml glass muscle bathing chamber which was maintained at 37°C, via a surrounding water jacket which was supplied by an accurately controlled water circulator.

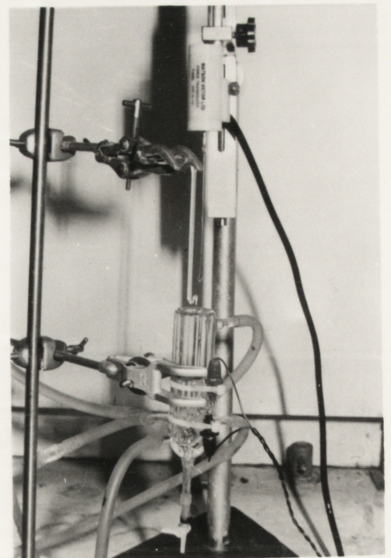
The muscle chamber had two gas inlets to the interior of the vessel. Either carbogen or halothane + carbogen would be supplied to the chamber through these inlets. A tap at the bottom of the chamber was used to facilitate quick and easy removal of used buffer (figure 7). The rate of gas delivery was about 100 ccs/minute of carbogen or halothane + carbogen both of which were controlled with

FIGURE 7

THE EQUIPMENT FOR TESTING IN VITRO MUSCLE RESPONSE
BEFORE THE ADDITION OF A MUSCLE FIBRE BUNDLE



1. VAPORISER
 2. TRANSDUCER
 3. MUSCLE CHAMBER
 4. AMPLIFIER
 5. RECORDER
 6. WATER CIRCULATOR
- HAL. = HALOTHANE INLET
CARB. = CARBOGEN INLET



AN ENLARGEMENT OF
THE MUSCLE CHAMBER

accurate flow meters.

The supply of carbogen was split into two streams. One stream went directly to the muscle bathing chamber, while the other was connected to a Dräger 'Vapor' halothane vaporiser, which was connected to the other gas inlet on the muscle chamber. A two-way gas valve was used to direct the main stream of carbogen either directly to the muscle chamber or to the halothane vaporiser. Two different circuits were used to eliminate the possibility of halothane contamination from gas tubing after a previous run with halothane (figure 7). The 'Vapor' actually delivered a very low concentration of halothane (0.1%) even when set on 0%, hence the need for an independent by-pass.

One of the rings from the fibre bundle was placed over a metal hook which was attached to a Watson-Victor force transducer. The transducer was fixed to a sliding block which could be gently raised or lowered. The other ring was fixed to a glass rod and the piece of muscle gently tensioned initially to less than one gram. The signal from the transducer was amplified by a Sanei strain gauge amplifier and monitored by a Perkin-Elmer continuous chart recorder. The system was calibrated by hanging appropriate weights on the transducer, and adjusting the pen travel on the recorder.

The muscle bathing chamber with 25 mls of standard buffer was raised to cover the muscle sample, recorder switched on and tension adjusted to one gram.

A steady baseline was obtained, and drugs were added to the buffer surrounding the muscle via disposable syringes or micro syringes depending on the volume to be added. If volumes greater than 1 ml were to be added to the bath, an equal volume of buffer was removed before the addition.

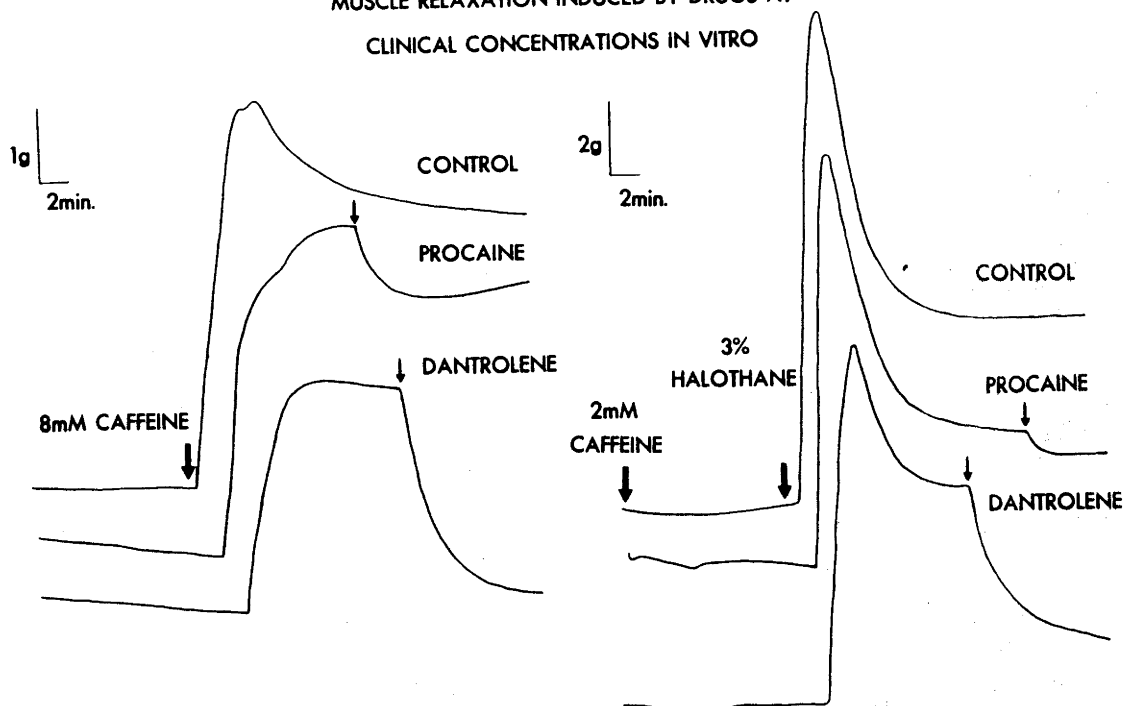
The system just described was duplicated and set up in a fume hood in order to eliminate halothane contamination of the laboratory atmosphere.

The duplication of the system served three main purposes. Firstly, the muscle bundles could be tested twice as quickly as a single system, hence reducing muscle aging to a minimum. Secondly, as a consequence of the decreased experimental time, two experiments were often performed during the day, thereby generating twice as much information. This was impossible when only a single system was used because it took up to 11-12 hours before all the fibre bundles had been tested. Thirdly, duplicate systems enabled better experimental quality control.

iv) Experimental variation and numerical analysis

The methods just described include two basic sources of variation. Fibre-fibre variation with fibres from the same patient was moderate but considered quite tolerable. This variation was thought initially to be due mainly to muscle deterioration over the six hours of a typical experiment. General muscle response fell progressively from the time of removal from the patient.

FIGURE 8
MUSCLE RELAXATION INDUCED BY DRUGS AT
CLINICAL CONCENTRATIONS IN VITRO



Procaine (0.5 mM) and dantrolene (6 μ M) were added to the muscle bath when the contractures had reached steady-state levels. The arrows indicate the points at which drugs were added to the normal human muscle preparations.

Patient-patient variation was a little more difficult to quantitate. The source of the variation appeared to be a real difference in sensitivity to caffeine or caffeine + halothane between patients.

The total amount of information generated during this study amounted to over thirty-five thousand (35,000) variables. Manipulation of the data by hand became a time consuming exercise, even after the completion of very few experiments. A comprehensive, versatile computer system was therefore developed to assist in the analysis of the data (see Appendix).

D: Results

i) Drug effects at clinical concentrations on normal human muscle

a) Drug induced relaxation

Examples of drug induced relaxation of caffeine contractures are shown in figure 8. After obtaining a baseline at a resting tension of about 1 gram, contracture was induced by the addition of 8 mM caffeine. Within one to two minutes a peak tension was reached which was followed by a short relaxation phase. Relaxation approached a steady-state level of tension after about four minutes, following the addition of the caffeine. If dantrolene or procaine were added at this point, immediate and sustained relaxation occurred. Relaxation induced by procaine was obviously not as great as that induced by dantrolene. Both drugs were at clinical concentration levels. None of the other drugs studied had a significant effect on relaxation.

TABLE 1

Muscle relaxation following drug treatments at clinical concentrations expressed as a percentage change in muscle tension

Drug	Concen- tration μ M	Concen- tration mg	Equivalent dosage mg	ADDED AFTER 8 mM CAFFEINE			ADDED AFTER 2 mM CAFFEINE + 3% HALOTHANE		
				Mean \pm S.E.M.	P	No. of fibres	Mean \pm S.E.M.	P	No. of fibres
Dantrolene	5.9		10	-107 \pm 8	-	5	-97 \pm 12	-	6
Procaine	500		680	-20 \pm 2	<0.0001	7	-11 \pm 1	<0.0005	5
Procainamide	500		679	-5 \pm 3	<0.0001	6	0 \pm 0.2	<0.0005	6
Hydrocortisone	55.2		100	-1 \pm 2	<0.0001	5	-1 \pm 1	<0.0005	5
Dexamethasone	7.75		20	+2 \pm 2	<0.0001	5	-2 \pm 2	<0.0005	5

P = Significance value from t-test (Results for each drug compared with the dantrolene results)
 S.E.M. = Standard error of the mean
 Equivalent dosage = Clinical dosage assuming blood volume of 5 litres
 Concentration = Final concentration in muscle bath
 A total of 55 muscle fibres from 19 normal subjects were tested in vitro.

Examples of typical contractures induced by caffeine potentiated halothane are also shown in figure 8. It can be seen that no contracture occurred upon addition of 2 mM caffeine. With normal muscle, contractures were not usually induced by halothane, but if caffeine (2 mM) was present in the muscle buffer, the addition of halothane (3%) resulted in a contracture of about 9 g.

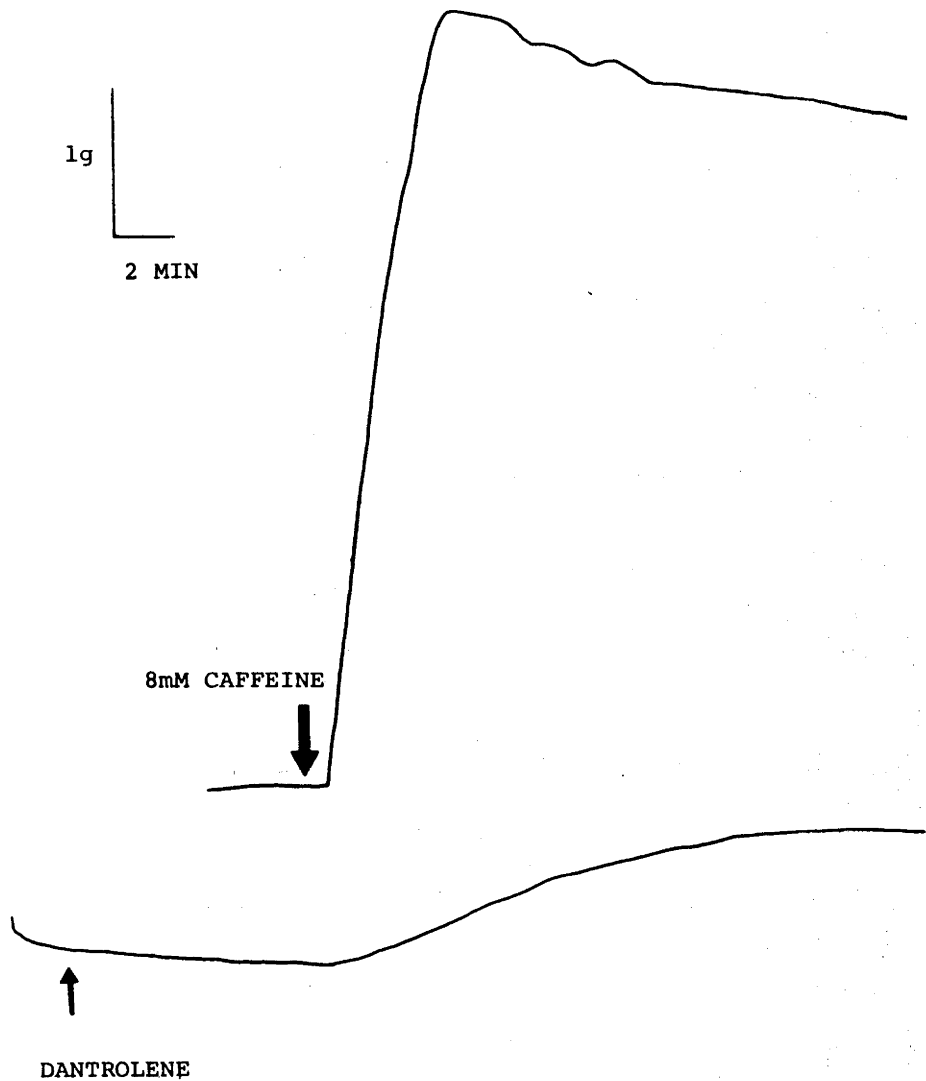
Appropriate drugs were added to the muscle bath during the relaxation steady-state level as before. The effect of dantrolene at a clinical concentration was dramatic. Almost complete relaxation was immediately induced and maintained. Procaine induced a small degree of relaxation. No other drug tested appeared to induce relaxation.

Each experiment was repeated with a minimum of five fibres and usually on more than three patients. A summary of the results obtained on all drugs tested is presented in table 1.

Relaxation is presented as the percentage drop in tension following the addition of a drug. Procainamide, hydrocortisone and dexamethasone failed to induce relaxation following either type of contracture. The P value was obtained by a t-test between the data from dantrolene and each of the other drugs. It can be seen from table 1 that the degree of relaxation induced by dantrolene is significantly greater than all the other drugs including procaine. The difference between the effect of dantrolene on caffeine contractures and caffeine + halothane contractures was not

FIGURE 9

INHIBITION OF AN 8mM CAFFEINE CONTRACTURE BY DANTROLENE
AT A CLINICAL CONCENTRATION IN VITRO



Dantrolene ($6 \mu\text{M}$) was added to the normal human muscle about 6 minutes before the caffeine.

TABLE 2

Inhibition of 8 mM caffeine contractures by drugs at clinical concentrations

Drug	TREATMENT		ADDED BEFORE 8 mM CAFFEINE			CONTROL	
	Concen- tration μ M	Equivalent dosage mg	Mean tension (g) \pm S.E.M.	No. of fibres	Mean tension (g) \pm S.E.M.	No. of fibres	P
Dantrolene	5.9	10	0.7 \pm 0.2	6	2.7 \pm 0.4	13	<0.01
Procaine	500	680	2.6 \pm 0.8	5	3.5 \pm 0.5	16	N.S.
Procainamide	500	679	2.5 \pm 0.9	5	2.8 \pm 0.6	11	N.S.
Hydrocortisone	55.2	100	3.8 \pm 0.9	5	3.6 \pm 0.5	7	N.S.
Dexamethasone	7.75	20	4.3 \pm 1.1	5	3.5 \pm 0.6	12	N.S.

P = Significance value from t-test
S.E.M. = Standard error of the mean
N.S. = Not significant

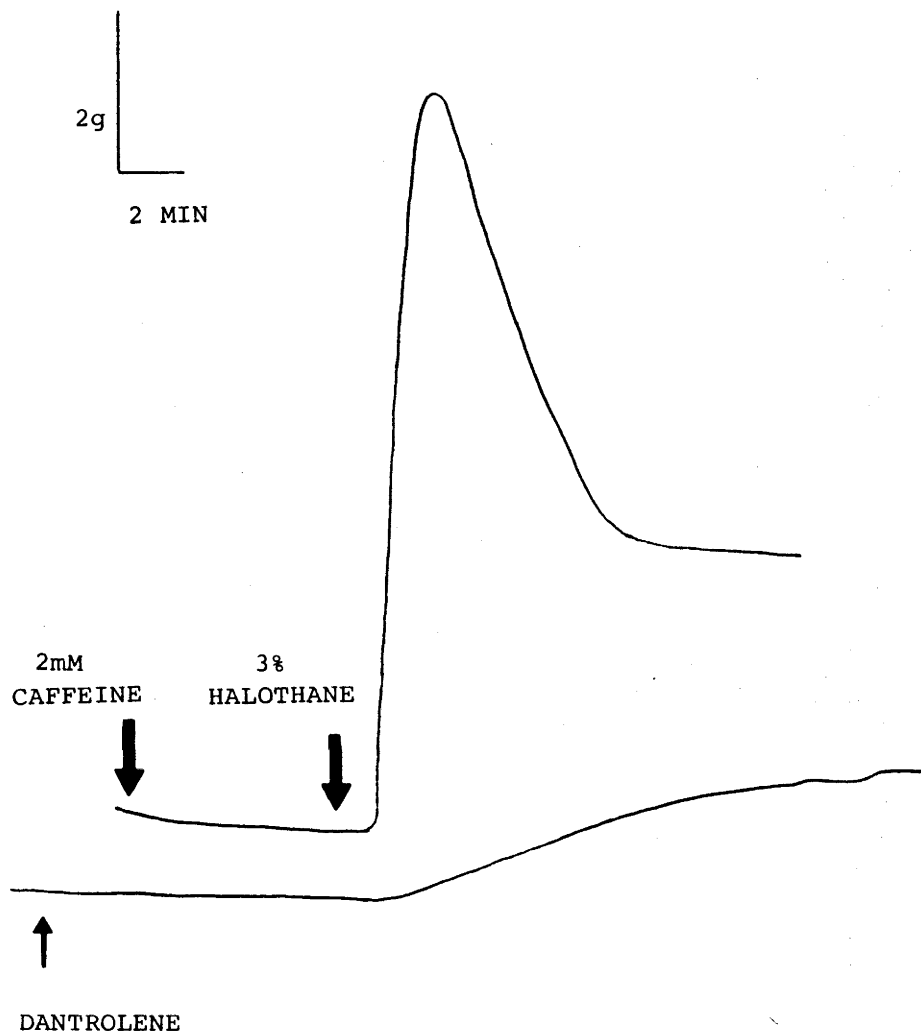
Equivalent dosage = Clinical dosage assuming blood volume of 5 litres

Concentration = Final concentration in muscle bath

A total of 85 muscle fibres from 19 normal subjects were tested in vitro.

FIGURE 10

INHIBITION OF A 2mM CAFFEINE + 3% HALOTHANE CONTRACTURE
BY DANTROLENE AT A CLINICAL CONCENTRATION IN VITRO



Dantrolene ($6 \mu\text{M}$) was added to the normal human muscle about 6 minutes before the caffeine.

TABLE 3

Inhibition of 2 mM caffeine + 3% halothane contractures by drugs at clinical concentrations

Drug	TREATMENT		ADDED BEFORE 2 mM CAFFEINE + 3% HALOTHANE			CONTROL	
	Concen- tration μ M	Equivalent dosage mg	Mean tension (g) \pm S.E.M.	No. of fibres	Mean tension (g) \pm S.E.M.	No. of fibres	P
Dantrolene	5.9	10	3.5 \pm 0.8	5	7.9 \pm 0.8	13	<0.01
Procaine	500	680	6.1 \pm 0.6	5	8.7 \pm 0.8	9	N.S.
Procainamide	500	679	6.3 \pm 1.5	5	7.2 \pm 0.9	13	N.S.
Hydrocortisone	55.2	100	10.8 \pm 1.0	7	12.0 \pm 1.3	8	N.S.
Dexamethasone	7.75	20	9.0 \pm 1.7	5	10.1 \pm 1.3	8	N.S.

P = Significance value from t-test
S.E.M. = Standard error of the mean
N.S. = Not significant

Equivalent dosage = Clinical dosage assuming blood volume of 5 litres
Concentration = Final concentration in muscle bath
A total of 78 muscle fibres from 10 normal subjects were tested in vitro.

statistically significant. Procaine, on the other hand, did have a greater effect on caffeine compared to caffeine + halothane contractures and this was significant at the 2% level.

b) Drug inhibition of 8 mM caffeine contractures

The inhibitory effect of a drug was measured by recording the tension developed by 8 mM caffeine in the presence and absence of the drug.

An example of the inhibitory effect of dantrolene on an 8 mM caffeine contracture is shown in figure 9. Dantrolene not only inhibited the peak tension produced but also prolonged the time to peak contracture.

The results for all drugs tested at clinical concentrations are presented in table 2. Dantrolene inhibited 8 mM caffeine contracture by about 75%. No other drug tested in this series significantly inhibited caffeine contractures.

c) Drug inhibition of 2 mM caffeine + halothane contractures

The inhibiting effect of dantrolene on a 2 mM caffeine potentiated halothane contracture is shown in figure 10. Pre-treatment of a fibre with dantrolene inhibits the caffeine + halothane contracture in normal human muscle by about 60%. No other drug suggested for the treatment of malignant hyperpyrexia at a clinical concentration had a significant effect on caffeine + halothane contractures (table 3). There was a strong suggestion that procaine

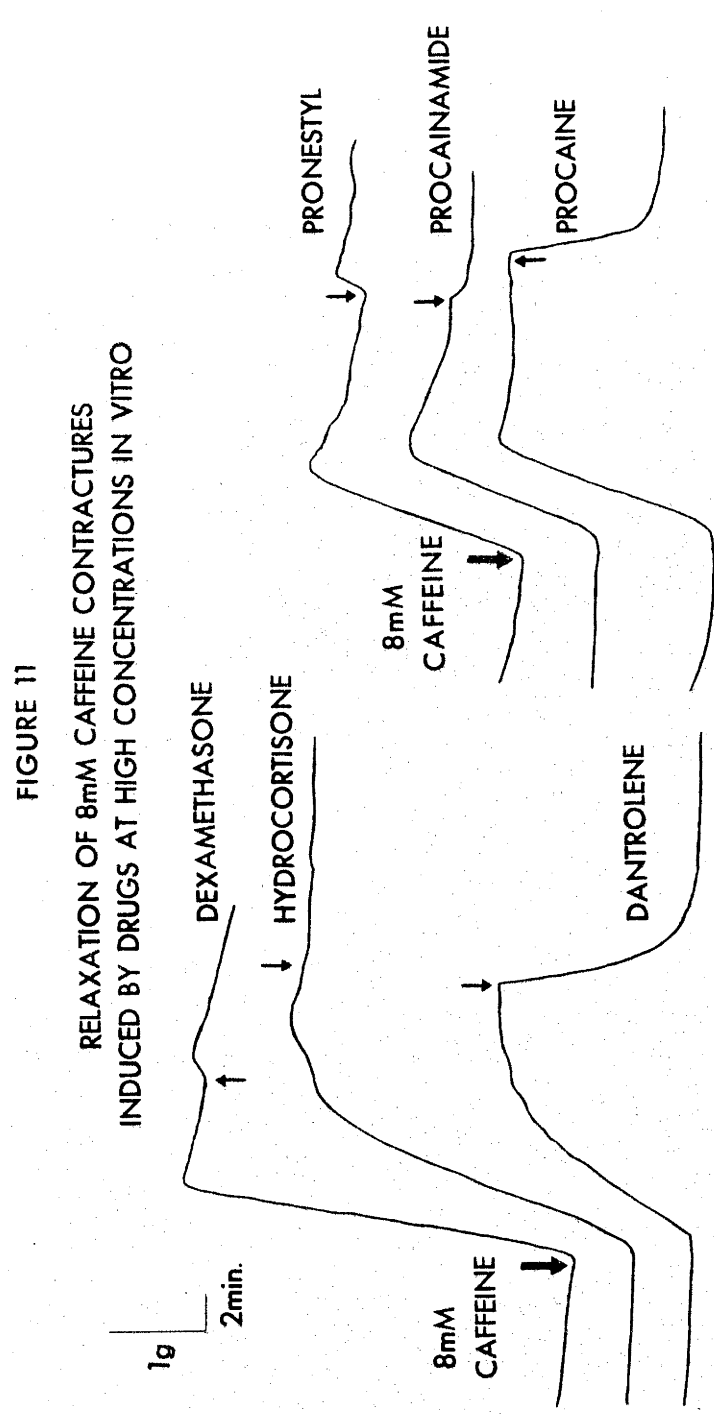
inhibited the contracture slightly (-20%) but this was not statistically significant ($P > 0.06$).

The apparent discrepancies between controls can be explained by the differences in caffeine and halothane sensitivity in different patients. Occasionally the two or three patients selected were more sensitive than usual to a particular drug combination, thus giving a high control mean.

ii) Drug effects at high concentrations on normal human muscle

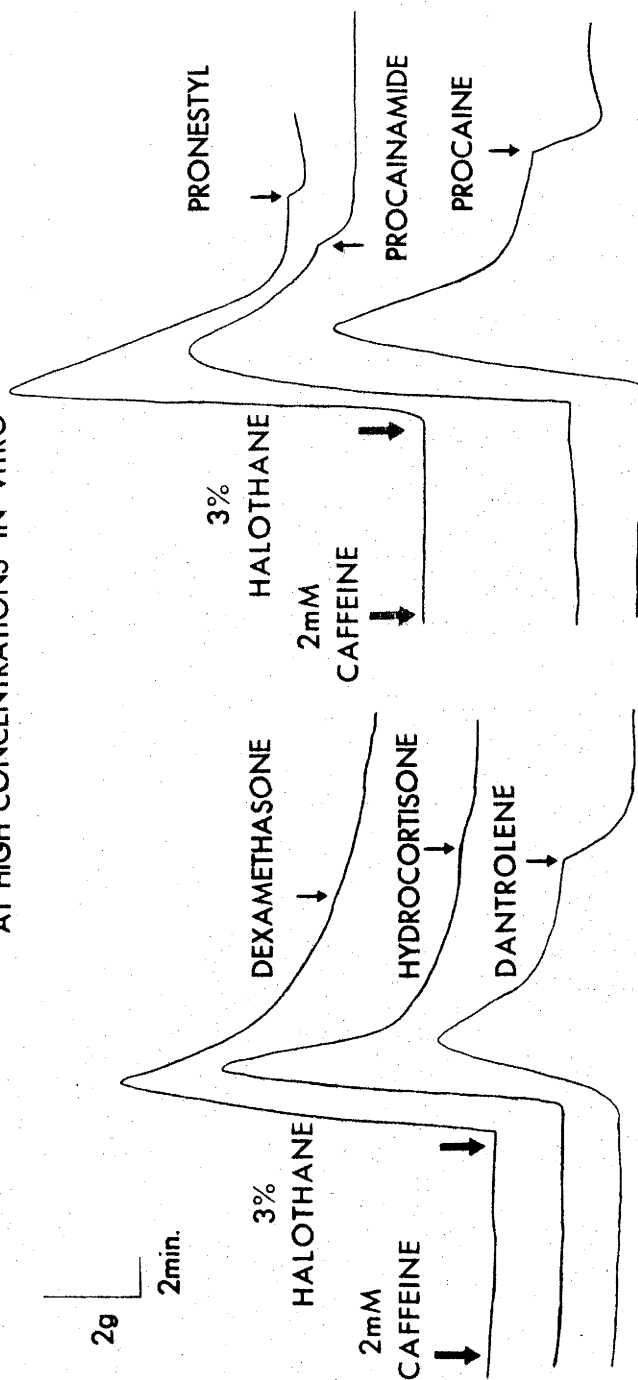
Drug concentrations used in this study were above the usual levels recommended clinically. The levels of hydrocortisone, dexamethasone and dantrolene may be achieved clinically but the concentrations of procaine, procainamide and pronestyl would be dangerous to attempt in vivo.

Pronestyl is a trade name for a clinical sample of procainamide. There are however, other additives present as discussed in the Materials and Methods section. The effect of the benzyl alcohol in the pronestyl mixture was investigated following reports that lower alcohols can potentiate the effects of local anaesthetics on muscle calcium stores (Grist, Hall and Baum, 1973; Clarke and Ellis, 1975; Ehrenpreis, 1965; Seeman, Chau, Goldberg, Sauks and Sax, 1971). Any difference between procainamide and pronestyl would be due to the preservatives added to the pronestyl.



The drugs being tested were added to the normal human muscle when the caffeine contracture had reached a steady-state level. The drugs used had the following concentrations: dexamethasone (77.5 μ M), hydrocortisone (552 μ M), dantrolene (30 μ M), pronestyl (5 mM), procainamide (5 mM) and procaine (5 mM).

FIGURE 12
RELAXATION OF 2mM CAFFEINE + 3% HALOTHANE CONTRACTIONS INDUCED BY DRUGS
AT HIGH CONCENTRATIONS IN VITRO



The drugs being tested were added to the normal human muscle when the caffeine + halothane contracture had reached a steady-state level. The drugs used had the following concentrations: dexamethasone (77.5 μ M), hydrocortisone (552 μ M), dantrolene (30 μ M), pronestyl (5 mM), procainamide (5 mM) and procaine (5 mM).

a) Drug induced relaxation

Typical results obtained with the six drugs studied on 8 mM caffeine induced contractures are presented in figure 11. Dantrolene immediately and completely reversed a caffeine contracture and the relaxation was sustained. Procaine also caused immediate relaxation, but the extent of reversal was less than for dantrolene. Although procainamide also caused relaxation its effect was not nearly as pronounced as procaine. Dexamethasone and hydrocortisone had no significant effect on caffeine contractures. Pronestyl caused a secondary contracture to take place on top of the caffeine steady-state tension. A subsequent study revealed that benzyl alcohol at the same levels present in pronestyl would produce a similar contracture. Benzyl alcohol did not induce a contracture in muscle that had not been treated with caffeine.

Examples of normal muscle reaction to the various drugs during caffeine potentiated halothane contractures are shown in figure 12. Although the results for dantrolene, procaine and procainamide appear similar to those obtained with caffeine induced contractures, the responses to the other drugs are different. Dexamethasone had no effect on tension response, hydrocortisone caused substantial relaxation, and pronestyl promoted the same degree of relaxation as procainamide.

A summary of the experiments on the effects of these drugs on muscle tension during caffeine and caffeine potentiated halothane contractures is shown in table 4.

TABLE 4

Muscle relaxation following drug treatments at high concentrations expressed as a percentage change in muscle tension

Drug	Concen- tration μM	Concen- Equivalent dosage mg	ADDED AFTER 8 mM CAFFEINE			ADDED AFTER 2 mM CAFFEINE + 3% HALOTHANE		
			Mean % \pm S.E.M.	P	No. of fibres	Mean % \pm S.E.M.	P	No. of fibres
Dantrolene	29.5	50	-103 \pm 3	-	6	-106 \pm 7	-	5
Procaine	5.0x10 ³	6800	-82 \pm 6	<0.03	8	-60 \pm 9	<0.01	5
Procainamide	5.0x10 ³	6790	-15 \pm 4	<0.0001	8	-20 \pm 7	<0.0005	8
Hydrocortisone	552.0	1000	-1 \pm 0.5	<0.0001	5	-33 \pm 6	<0.0005	6
Dexamethasone	77.5	200	+7 \pm 2	<0.0001	5	-4 \pm 2	<0.0001	5
Pronestyl	5.0x10 ³	6790	+31 \pm 7	<0.0001	11	-21 \pm 3	<0.0001	7

P = Significance value from t-test (Results for each drug compared with the dantrolene results)
 S.E.M. = Standard error of the mean
 Concentration = Final concentration in muscle bath
 Equivalent dosage = Clinical dosage assuming blood volume of 5 litres
 A total of 79 muscle fibres from 37 normal subjects were tested in vitro.

Relaxation is presented as a percentage drop in muscle tension in relation to tension developed just before the addition of the drug. Dantrolene usually promoted relaxation to such an extent that the final tension was often below the initial resting tension.

The results for each drug have been compared with those obtained with dantrolene by the Student's t-test. Dantrolene was significantly more efficient in promoting muscle relaxation for both types of contracture when compared with all the other drugs (table 4).

Apart from the results obtained with dantrolene, two results require further emphasis. The augmenting effect of pronestyl on 8 mM caffeine contractures contrasts with a substantial relaxing effect of pronestyl when added to caffeine potentiated halothane contractures.

The addition of hydrocortisone to the muscle bath during a caffeine potentiated halothane contracture, resulted in the muscle tension being reduced by about 33%. In contrast, hydrocortisone appeared to have no relaxing effect on caffeine contractures (table 4).

b) Drug inhibition of 8 mM caffeine contractures

The effect of procaine and dantrolene on the development of 8 mM caffeine contractures is shown in figure 13. None of the other four drugs tested had any effect on the tension developed by the fibres. Both dantrolene and procaine prolonged the onset of contracture and the size

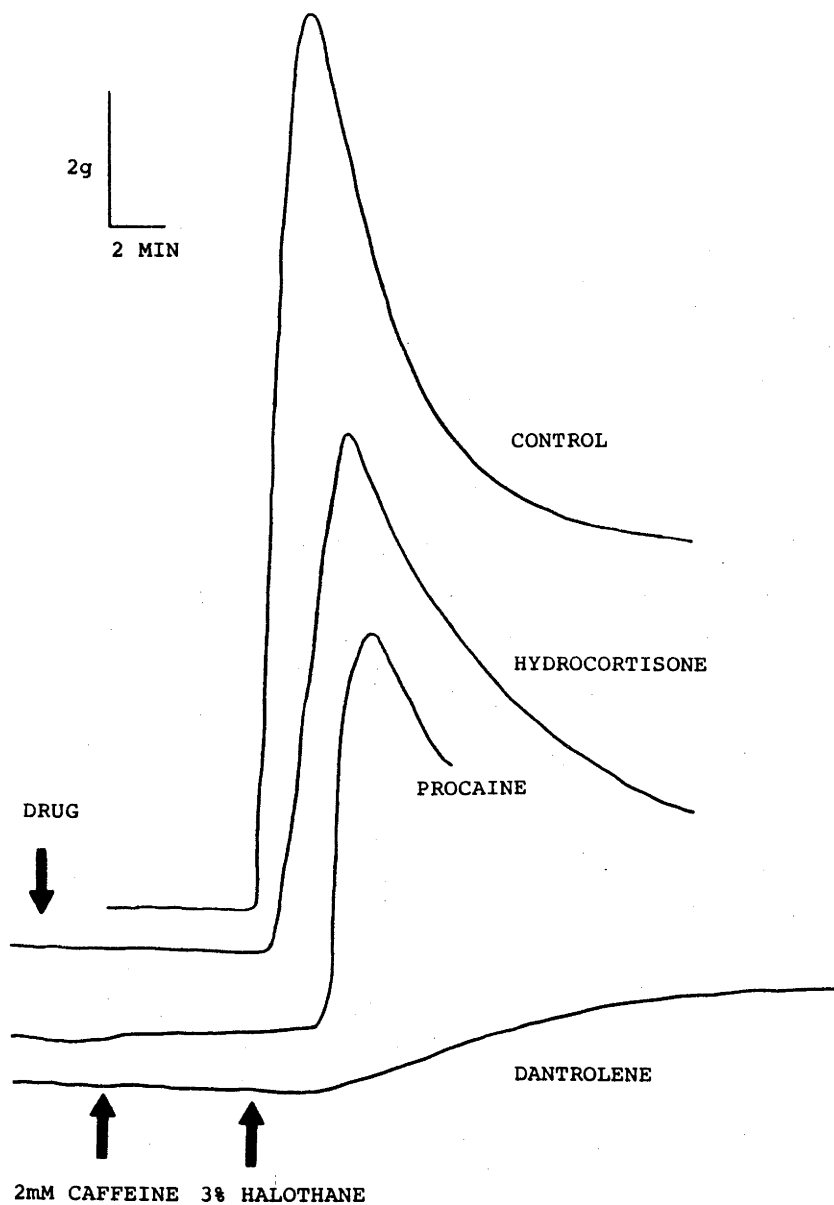
TABLE 5
Inhibition of 8 mM caffeine contractures by drugs at high concentrations

TREATMENT			ADDED BEFORE 8 mM CAFFEINE		CONTROL	
Drug	Concen- tration µM	Equivalent dosage mg	Mean % ± S.E.M.	No. of fibres	Mean % ± S.E.M.	No. of fibres P
Dantrolene	29.5	50	0.5±0.2	7	2.5±0.4	12 <0.004
Procaine	5.0x10 ³	6800	0.5±0.2	5	3.5±0.8	10 <0.03
Procainamide	5.0x10 ³	6790	4.8±1.1	6	3.9±0.9	11 N.S.
Hydrocortisone	552.0	1000	4.2±1.2	5	3.6±0.4	15 N.S.
Dexamethasone	77.5	200	3.6±0.7	5	2.5±0.6	15 N.S.
Pronestyl	5.0x10 ³	6790	3.6±0.6	6	2.7±0.4	10 N.S.

P = Significance value from t-test
S.E.M. = Standard error of the mean
N.S. = Not significant
Equivalent dosage = Clinical dosage assuming blood volume of 5 litres
Concentration = Final concentration in muscle bath
A total of 107 muscle fibres from 20 normal subjects were tested in vitro.

FIGURE 14

INHIBITION OF 2mM CAFFEINE + 3% HALOTHANE CONTRACTURES
BY DRUGS AT HIGH CONCENTRATIONS IN VITRO



Hydrocortisone (552 μ M), procaine (5 mM) and dantrolene (30 μ M) were added to the normal human muscle about 6 minutes before the caffeine.

of the peak tension. The inhibitory effect on absolute peak tension produced was similar for the two drugs. A summary of the results of peak tensions produced by 8 mM caffeine following pre-treatment of the muscle with each drug is presented in table 5.

The inhibitory effects of dantrolene and procaine are similar, both reducing caffeine contractures to between 15 to 20% of the tension developed in the absence of the drug. Although tension figures presented in table 5 would appear to suggest a potentiation of 8 mM caffeine contractures by pronestyl, the difference between the means was not statistically significant. Neither hydrocortisone, procainamide, pronestyl, nor dexamethasone treatments were statistically different from the appropriate controls (table 5).

c) Drug inhibition of 2 mM caffeine + 3% halothane contractures

Some of the inhibitory effects at the higher drug concentrations studied are shown in figure 14. The caffeine potentiated halothane contracture is a particularly severe test for the efficiency of any drug to inhibit. Dantrolene was the most efficient drug in inhibiting such contractures.

Hydrocortisone had a substantial inhibitory effect on caffeine potentiated halothane contractures, although to a lesser extent than dantrolene or procaine.

TABLE 6

Inhibition of 2 mM caffeine + 3% halothane contractures by drugs at high concentrations

Drug	TREATMENT		ADDED BEFORE 2 mM CAFFEINE + 3% HALOTHANE		CONTROL	
	Concen- tration μ M	Equivalent dosage mg	Mean % \pm S.E.M.	No. of fibres	Mean % \pm S.E.M.	No. of fibres P
Dantrolene	29.5	50	2.0 \pm 0.8	5	7.5 \pm 0.8	11 <0.002
Procaine	5.0x10 ³	6800	2.6 \pm 0.5	6	7.3 \pm 0.9	13 <0.006
Procainamide	5.0x10 ³	6790	9.1 \pm 2.8	6	8.9 \pm 1.3	10 N.S.
Hydrocortisone	552.0	1000	5.6 \pm 0.9	7	9.7 \pm 1.3	11 <0.05
Dexamethasone	77.5	200	6.8 \pm 1.2	7	8.0 \pm 0.9	12 N.S.
Pronestyl	5.0x10 ³	6790	4.5 \pm 0.8	4	5.2 \pm 1.0	5 N.S.

P = Significance value from t-test
 S.E.M. = Standard error of the mean
 N.S. = Not significant

Equivalent dosage = Clinical dosage assuming blood volume of 5 litres
 Concentration = Final concentration in muscle bath
 A total of 97 muscle fibres from 19 normal subjects were tested in vitro.

The relative efficacy of all drugs tested can be seen in table 6. In contrast to the results presented for caffeine induced contractures in normal muscle, three results were significant.

Dantrolene inhibited the caffeine potentiated halothane contracture routinely by about 75%, procaine by about 65%, and hydrocortisone by approximately 40%. Procainamide and pronestyl did not appear to affect muscle contracture.

E: Discussion

Results comparing the relative efficacy of five drugs in inhibiting and relaxing caffeine and caffeine + halothane contractures in vitro, in normal human muscle have been described. Each of the five drugs has been suggested as a suitable treatment for malignant hyperpyrexia. However most proposals have been empirical (see Section I, D; British Medical Journal, 1968; Harrison, 1971; Ellis, Clarke, et. al., 1974). The efficacy of a particular drug in inhibiting or relaxing caffeine or halothane induced contractures in malignant hyperpyrexia muscle is the main basis used to justify a specific treatment (Ellis, Clarke, et. al., 1974; Moulds and Denborough, 1972; Ellis, Keany and Harriman, 1973).

The shortage of human malignant hyperpyrexia muscle for experimental studies necessitated the use of a normal muscle model. The justification of the model with normal muscle was as follows:

1. Drugs that have been shown to reverse halothane contractures in malignant hyperpyrexia muscle have similar effects on contractures induced in the normal muscle model.
2. Malignant hyperpyrexia muscle is particularly sensitive to caffeine, halothane and caffeine potentiated halothane contractures. Similar types of contracture can be simulated in

normal muscle, by either increasing the concentration of caffeine, or by pre-treating muscle with low levels of caffeine and then exposing the muscle to halothane.

3. The mechanism of the abnormal release of calcium into the myoplasm which occurs using these agents, appears to be similar for both types of muscle.

The results presented in table 1, show that dantrolene and procaine were the only drugs tested at usual clinical levels, that caused significant relaxation of 8 mM caffeine contractures. Dantrolene was far more effective in promoting relaxation than procaine (-107% compared to only -20%, table 1). A similar result was obtained if relaxation of caffeine + halothane contractures was used as the model (-97% compared to only -11% table 1). Reports on the efficacy of procaine in antagonising muscle contracture in normal and malignant hyperpyrexia muscle have involved very high dosage levels (see Section I, D, Clarke and Ellis, 1975; Moulds and Denborough, 1972). Moulds and Denborough (1972), suggested that the procaine effect was dose dependent and that lower levels of procaine at clinical concentrations may be effective. The results presented in table 1 appear to be the first reported effects at therapeutic levels, of procaine on caffeine and caffeine + halothane contractures in normal human muscle (Clarke and Ellis, 1975; Hall and Lister, 1974).

Although procaine failed to inhibit the onset of either type of contracture (tables 2 and 3), its effect in promoting relaxation may still be relevant to the treatment of malignant hyperpyrexia once the syndrome has been triggered (table 1). However, any academic discussion involving the usefulness of procaine as a treatment for malignant hyperpyrexia is overshadowed by the dramatic effects of dantrolene (tables 1, 2 and 3). Dexamethasone, hydrocortisone and procainamide did not have any effect on inhibiting or relaxing either type of contracture (tables 1, 2 and 3).

Similar experiments to those just discussed but with higher drug concentrations are summarised in tables 4 to 6.

Dantrolene appeared to be no more effective at the higher concentration than at the lower level. This is not surprising as total relaxation of both types of contracture occurred at the lower drug level.

Procaine, on the other hand, showed a marked increase in its relaxation effect at the higher dose (compare table 1 with table 4). Dantrolene was equally effective in inducing relaxation with both types of contracture, but procaine was always more effective in reversing caffeine contractures than caffeine + halothane contractures (table 4).

Procainamide, in contrast was usually more effective in reversing a caffeine + halothane contracture

than a caffeine contracture.

Even though procainamide had no effect in causing relaxation of either type of contracture at the clinical dose, an effect could be demonstrated at the higher dose (table 4). The degree of relaxation of caffeine contractures was appreciably less with procainamide than with procaine (-15% compared with -82%, table 4).

The only drug which was not effective in inhibiting caffeine contractures at the normal clinical concentration and which became effective at the higher level, was procaine (tables 2 and 5). Although the extent of suppression at 5 mM would infer that lower quantities of procaine might restrain caffeine contractures, 0.5 mM procaine failed to impair contracture development (table 2). A blood level of 0.5 mM is relatively high for clinical use and would be difficult to exceed. Hydrocortisone, dexamethasone and pronestyl failed to suppress or reverse caffeine contractures even at elevated concentrations (tables 4 and 5).

Dexamethasone also had no detectable effect on caffeine + halothane contractures (table 4). Although procaine appears superior to hydrocortisone in suppressing caffeine + halothane contractures, 5mM procaine is a toxic level, whereas 552 μ M hydrocortisone may be possible to obtain in vivo (table 6).

The relevance of all drugs tested, except dantrolene, in the treatment of malignant hyperpyrexia is doubtful.

Although procaine was a powerful inhibitor of caffeine and caffeine + halothane contractures at excessive therapeutic levels, clinical levels had very little effect.

Hydrocortisone did influence muscle tension at the higher level studied. At a concentration of 552 μ M, which may be possible to obtain in vivo, it hampered the onset of caffeine + halothane contractures as well as inducing considerable relaxation. It failed to alter muscle tension with caffeine alone as the stimulant, so its use in the prevention or treatment of malignant hyperpyrexia therefore depends on which normal muscle model is the more relevant. Two points should be stressed, however; firstly, hydrocortisone is more effective in antagonising muscle contracture than dexamethasone, and secondly the potency of dantrolene overshadows any effect by hydrocortisone.

Procainamide failed to alter muscle response when administered at clinical concentrations in vitro. Even at levels far in excess of those possible to obtain clinically, only relatively small relaxation responses were detectable and no inhibitory activity could be demonstrated. The alcohol present in pronestyl actually augmented caffeine contractures. Preservatives added to commercial drug preparations that are presented in soluble form may cause an adverse response in vivo. The preservatives present in dexamethasone may account for the difference in muscle response in vitro between it and hydrocortisone. The relative efficacy of procaine and procainamide in interfering with muscle contracture in vitro, would support procaine.

as the more appropriate drug in any treatment regime. If, for example, procaine or procainamide were to be used to control cardiac arrhythmia, procaine would be the better choice because of its added effect on skeletal muscle metabolism.

The observations just described imply that dantrolene would be the drug of choice in the treatment of malignant hyperpyrexia. At easily obtained blood levels, it completely reversed 8 mM caffeine as well as caffeine + halothane contractures; suppressed the induction of 8 mM caffeine by ~75% and caffeine + halothane contractures by ~60%; and was far more effective than any other drug suggested for the treatment of malignant hyperpyrexia at equivalent concentrations.

The most relevant model for malignant hyperpyrexia using normal muscle is difficult to ascertain. Both caffeine contractures as well as caffeine potentiated halothane contractures have been suggested. Evidence available at present suggests that caffeine and halothane may be acting at two distinct sites in human muscle (Nelson and Denborough, 1976, paper in preparation). Since the location of the lesion in malignant hyperpyrexia muscle is unknown, the significance of any differences of one model over the other is unclear. Nevertheless, the potency of dantrolene is both highly impressive and unprecedented.

The capability of dantrolene to inhibit both types of contracture suggests it may be extremely effective in

preventing the triggering of malignant hyperpyrexia in vivo. If this is the case, a great range of general anaesthetics become available for use in surgery on malignant hyperpyrexia patients. At present anaesthetists are limited in the choice of agents that can be used. Anaesthetic agents thought to have been safe in the past may induce a reaction in some patients as shown recently by Ellis et al. (1975). Pre-treatment of the patient with dantrolene before anaesthesia may render such drugs safe. The finding that dantrolene completely reversed muscle tension and sustained the relaxation produced implies it may also be used in the treatment of malignant hyperpyrexia.

SECTION III - DRUG EFFECTS ON HUMAN MALIGNANT

HYPERPYREXIA MUSCLE

A: Introduction

The opportunity to confirm the selection of the most effective drug in the treatment of malignant hyperpyrexia came at the conclusion of the experimental program just described. A serum creatine phosphokinase estimation was performed on a thirty-five year old male patient, whose brother had died from malignant hyperpyrexia, and found to be raised.

Raised serum creatine phosphokinase levels provide a relatively good indication of susceptibility to malignant hyperpyrexia, although occasional reports of normal levels in susceptible individuals have occurred (Ellis, Clarke, Modgill, Currie and Harriman, 1975). The raised creatine phosphokinase in this patient warranted further investigation, particularly when his family history was considered. The patient therefore returned to Canberra Hospital to have the definitive in vitro muscle test as described by Moulds and Denborough (1974c).

The first few muscle fibre bundles tested confirmed that the patient was susceptible to malignant hyperpyrexia. Some of the remaining fibres were then used to test the efficacy of some of the drugs tested earlier, in controlling the onset and relaxation of halothane and caffeine induced contractures.

B: Materials and methods

The materials and methods were basically the same as those described for normal muscle. The muscle clamp and stick technique was used as with normal muscle but the muscle biopsied was vastus lateralis (left leg). Two clamps were used in order to obtain as many fibre bundles as possible. The large muscle bulk of his thigh made this possible with little discomfort to the patient. The operation was performed under local anaesthesia (procaine) in case the patient was susceptible to malignant hyperpyrexia. Procaine was selected because of its demonstrated safety in malignant hyperpyrexia as opposed to lignocaine, which has been shown to enhance calcium release from the sarcoplasmic reticulum in vitro (Moulds et al., 1972). Care was taken by the surgeon not to inject procaine into the muscle sheath.

C: Results

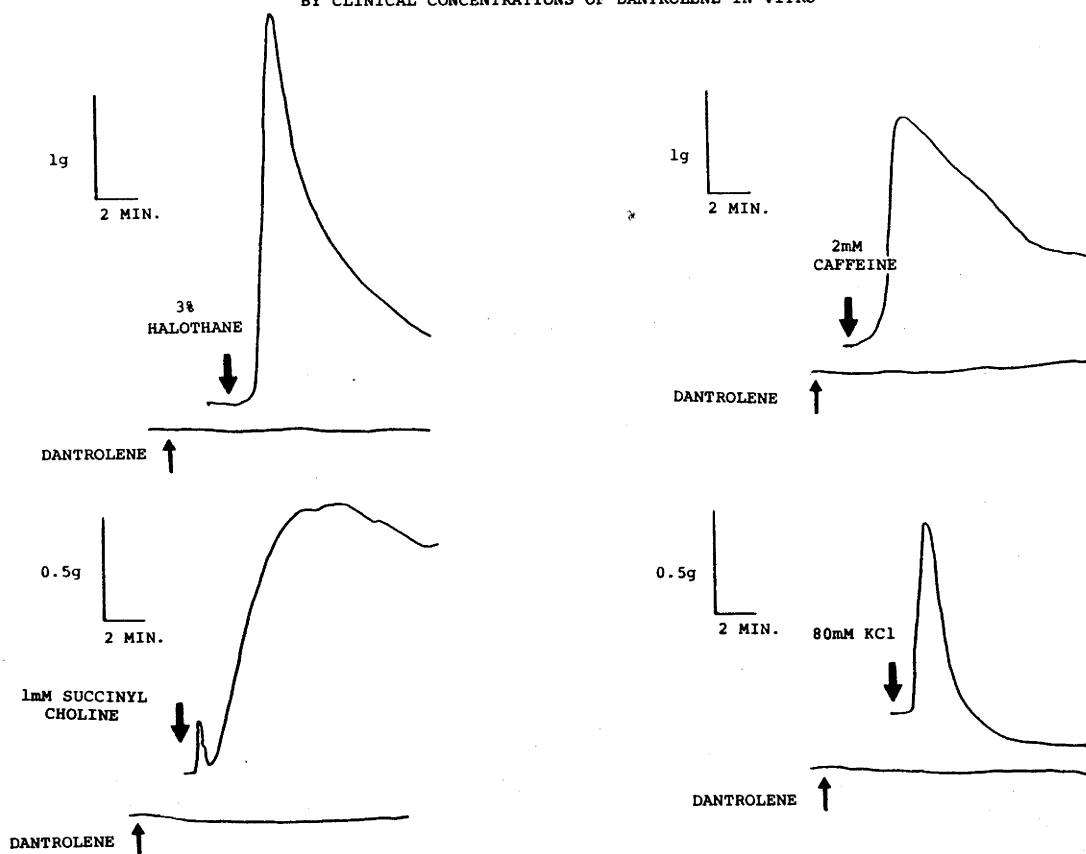
i) Confirmation of patient susceptibility to malignant hyperpyrexia

Succinylcholine, a muscle relaxant often used before general anaesthesia to facilitate intubation, is a potent precipitator of malignant hyperpyrexia in humans and swine (Hall, et al., 1966). Although this agent induces an in vitro muscle contracture in susceptible individuals, it has no effect on normal muscle (Moulds and Denborough, 1974a and b).

Depolarization of the sarcolemma membrane may be artificially induced by the rapid addition of potassium chloride to the muscle buffer. A concentration of 60 mM

FIGURE 15

INHIBITION OF MALIGNANT HYPERTHERMIA MUSCLE CONTRACTURES
BY CLINICAL CONCENTRATIONS OF DANTROLENE IN VITRO



Dantrolene ($6 \mu\text{M}$) was added to the human malignant hyperpyrexia muscle about 6 minutes before the drug being tested. The four treatments shown above do not induce a response in normal muscle.

potassium chloride ensures complete depolarization in frog muscle (Luttgau and Oetliker, 1968). A level of 80 mM potassium chloride when added to normal human muscle however fails to cause a contraction (Moulds and Denborough, 1974b). If human malignant hyperpyrexia muscle is treated with 80 mM potassium chloride an immediate contraction takes place.

The fact that low levels of caffeine (<2 mM) do not cause normal muscle to contract in vitro, has been discussed in previous sections. Occasionally small contractures could be induced by 3% halothane in normal muscle; however they were seldom over 0.2 g.

Confirmation of the susceptibility of the patient to malignant hyperpyrexia was obtained following tests on four fibres (figure 15). Large spontaneous contractures occurred when the fibres were exposed to halothane (3%), potassium chloride (80 mM), succinylcholine (1 mM) and a low level of caffeine (2 mM) (figure 15). Treatment of normal muscle with the same agents causes no effect (Moulds and Denborough, 1974b).

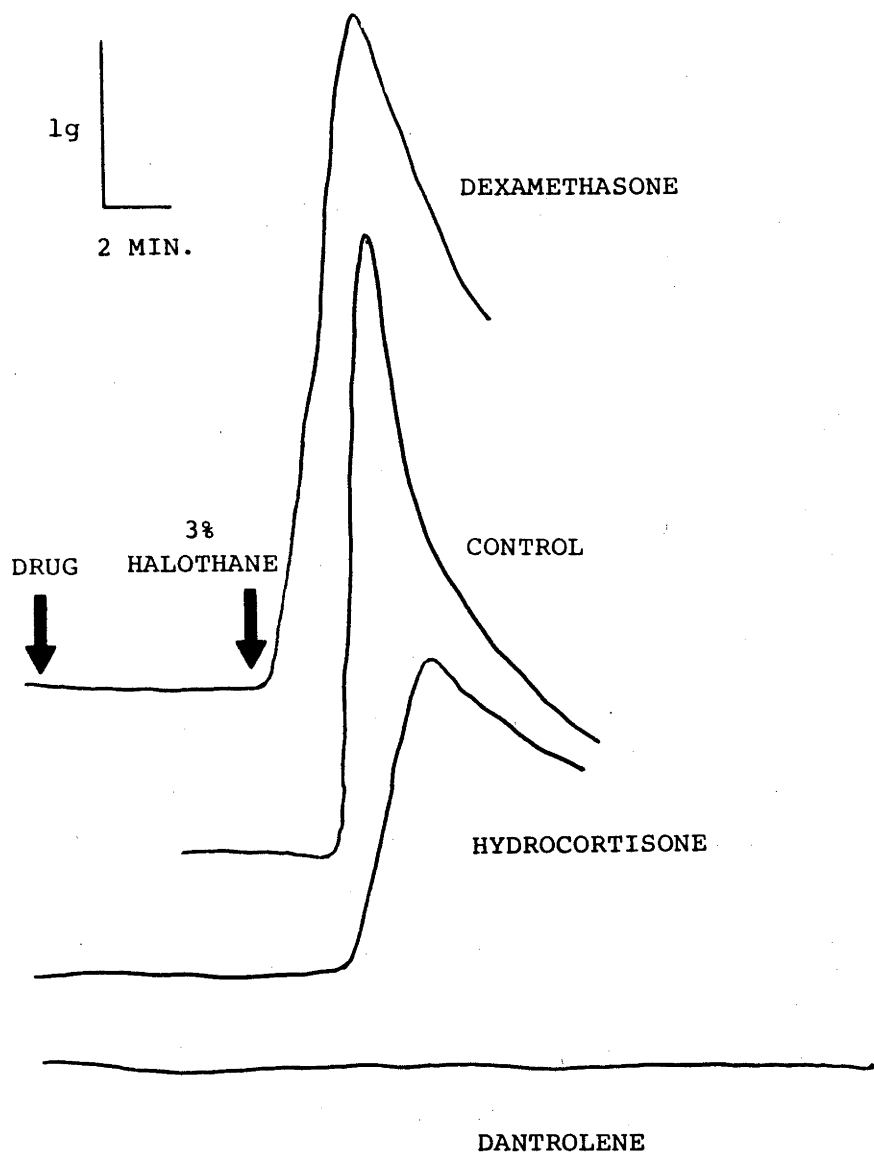
Total inhibition of all four types of contracture by dantrolene at a clinical concentration is also shown in figure 15.

ii) Drug inhibition of malignant hyperpyrexia muscle contracture

The comparative effects of dantrolene at a clinical concentration and hydrocortisone and dexamethasone at high

FIGURE 16

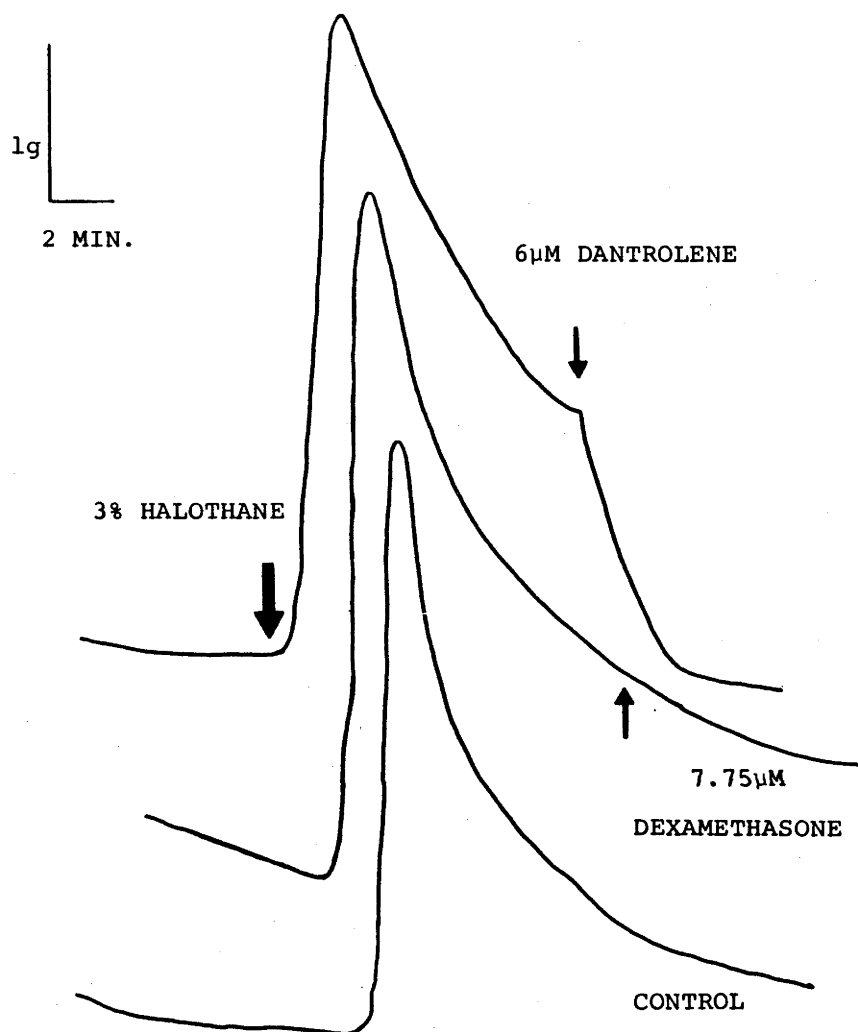
INHIBITION OF HALOTHANE CONTRACTURES
IN MALIGNANT HYPERTHYREXIA MUSCLE IN VITRO



Dexamethasone ($7.75 \mu\text{M}$), hydrocortisone ($55.2 \mu\text{M}$) and dantrolene ($6 \mu\text{M}$) were added to the human malignant hyperpyrexia muscle about 6 minutes before the halothane.

FIGURE 17

RELAXATION OF HALOTHANE CONTRACTURES IN MALIGNANT
HYPERPYREXIA MUSCLE BY CLINICAL CONCENTRATIONS OF DRUGS IN VITRO



The drugs being tested were added to the human malignant hyperpyrexia muscle when the halothane contracture had reached a constant rate of relaxation.

concentrations, in inhibiting halothane induced contractures can be seen in figure 16.

Dantrolene was the most efficient drug tested in inhibiting halothane induced contracture of malignant hyperpyrexia muscle (figure 16). Clinical (not shown) and high concentrations of hydrocortisone appeared to have similar inhibitory effects on halothane contractures. This lack of dose dependency by hydrocortisone requires further investigation as these treatments were not repeated. Dexamethasone, at relatively high levels, failed to inhibit a halothane induced contracture, in contrast to hydrocortisone. The suppression of halothane contractures by dantrolene overshadows the moderate response induced with hydrocortisone.

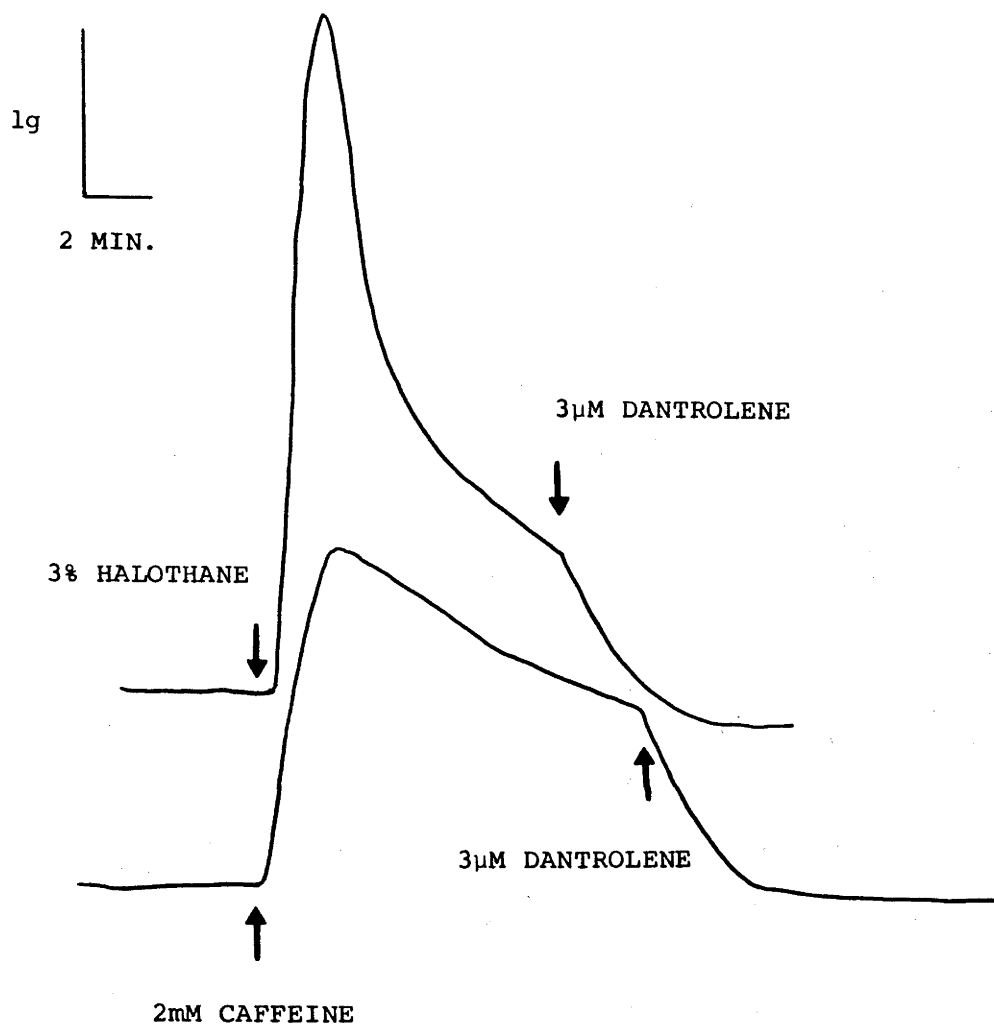
iii) Drug induced relaxation of malignant hyperpyrexia muscle

Unfortunately, due to lack of fibres, the efficacy of only two drugs in relaxing halothane induced contractures in malignant hyperpyrexia muscle could be tested. Dantrolene at a clinical level caused total, immediate and sustained relaxation to halothane induced muscle contracture (figure 17). Dexamethasone, at clinical levels however failed to alter the rate of relaxation during a similar contracture (figure 17).

The concentration of dantrolene used on malignant hyperpyrexia muscle was at a clinical level, but the efficacy of the drug warranted investigation at even lower concentrations. The reversal of a 3% halothane contracture and a 2 mM caffeine contracture by 3 μ M dantrolene is shown in figure 18.

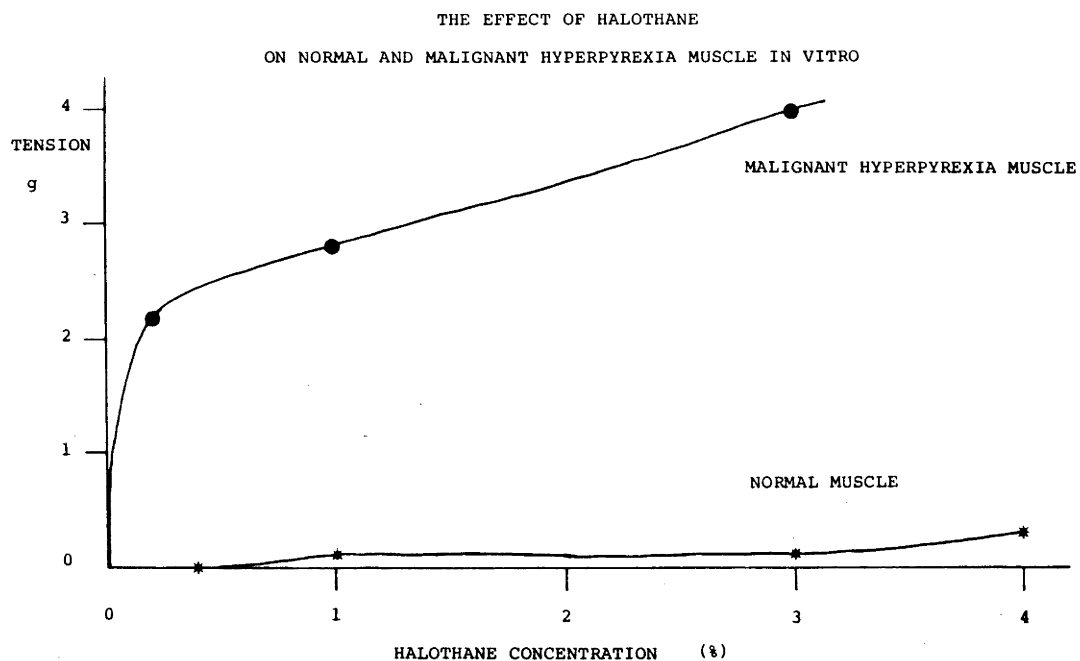
FIGURE 18

DRUG INDUCED RELAXATION OF MALIGNANT HYPERTHERMIA
MUSCLE CONTRACTURES BY LOW CONCENTRATIONS
OF DANTROLENE IN VITRO



The dantrolene was added to the human malignant hyperthermia muscle when the contracture had reached a constant rate of relaxation.

FIGURE 19



A total of 132 muscle fibres (Rectus Abdominis) from 58 normal subjects and 6 fibres (Vastus Lateralis) from a patient susceptible to malignant hyperpyrexia were tested in vitro. The number of observations were as follows: normal muscle, 4% (11), 3% (60), 1% (7), 0.4% (54); malignant hyperpyrexia muscle, 3% (4), 1% (1), 0.2% (1).

iv) Halothane sensitivity of malignant hyperpyrexia muscle

Muscle from patients susceptible to malignant hyperpyrexia is much more sensitive to halothane than normal muscle (figure 19). Perhaps a lower concentration of halothane could be used in future investigations. The results demonstrate the extreme sensitivity of malignant hyperpyrexia muscle to very low levels of halothane.

D: Discussion

Drug efficacy in suppressing and relaxing halothane and caffeine contractures in muscle from a patient susceptible to malignant hyperpyrexia has been measured. Contractures induced by halothane, succinylcholine, potassium chloride and caffeine were all abolished by low levels of dantrolene (6 μ M). Evidence available for its action on human muscle suggests that dantrolene may act at two sites, depending on its concentration (Nelson and Denborough, 1976, paper in preparation). At low concentrations (<3 μ M) it affects the excitation-contraction coupling apparatus, while at high levels (>6 μ M) also acts on the sarcoplasmic reticulum. The obliteration of the large halothane and succinylcholine contractures by dantrolene is striking. This is the first reported effect of dantrolene at clinical levels on halothane or succinylcholine contractures induced in human malignant hyperpyrexia muscle in vitro. The overwhelming confinement of contractures induced by two of the most potent precipitators of malignant hyperpyrexia in vivo, by dantrolene is impressive. This drug was equally effective in preventing and reversing halothane contractures (figures 15 and 17).

By contrast the results for dexamethasone and hydrocortisone were variable and unimpressive. Dexamethasone at normal clinical levels failed to alter the relaxation phase of a halothane contracture, and even at a high concentration had no inhibitory action. Hydrocortisone however did inhibit a halothane contracture by about 40%, but no dose dependent effect could be demonstrated. Relaxation of halothane induced muscle contractures by hydrocortisone was not measured because of the lack of material. One fibre pre-treated with low concentrations of dexamethasone gave a relatively poor response, however, this was thought to be due to a poor fibre rather than a real effect. The treatment at the highest level of dexamethasone lends support to this conclusion, since no inhibitory action was detected.

Extensive studies on the site of action and therapeutic value of dantrolene have been reported in recent years (Gilly and Costantin, 1974; Putney and Bianchi, 1974). Studies with dantrolene on human muscle suggest that at low concentrations ($<3 \mu\text{M}$) its main site of action is the excitation-contraction coupling mechanism in the sarcolemma. At higher concentrations an effect on the sarcoplasmic reticulum can also be demonstrated (Nelson and Denborough, 1976, paper in preparation).

Levels of dantrolene that inhibited the excitation-contraction coupling mechanism failed to suppress a halothane contracture in malignant hyperpyrexia muscle. Concentrations of dantrolene that inhibited caffeine contractures in normal

muscle completely suppressed halothane contractures in malignant hyperpyrexia muscle, lending support to a sarcoplasmic reticulum defect. Spontaneous contraction induced by the addition of potassium chloride and succinylcholine however imply that the sarcolemma is also affected.

The completeness of the relaxation and inhibition by dantrolene of halothane induced contractures in muscle from a susceptible patient supports its use in the treatment of malignant hyperpyrexia in vivo.

SECTION IV - GENERAL DISCUSSION

The study presented in this thesis was undertaken to find the most suitable drug treatment for malignant hyperpyrexia. Methods employed included in vitro pharmacologic models of malignant hyperpyrexia based on normal human muscle. The efficacy of drugs suggested for the treatment of malignant hyperpyrexia was evaluated by measuring muscle response to drug administration in vitro. Muscle response to the drugs not only suggests a new drug treatment regime for malignant hyperpyrexia but also suggests specific biochemical changes in malignant hyperpyrexia muscle membranes. The aetiology of malignant hyperpyrexia appears to include the detailed molecular structure and association of the sarcoplasmic reticulum with the sarcolemma.

A: Membrane abnormalities in malignant hyperpyrexia muscle

Significant advances in membrane structure in recent years have led to a more sophisticated understanding of the structure and function of muscle cell membranes. The sarcolemma, its invaginations and the sarcoplasmic reticulum are excitable membranes.

Excitable membranes possess different levels of excitation or energisation. Phospholipid and its fluidity in a membrane play an important role in membrane function and probably in maintenance of the energised state (Luria, 1975; Tasaki, 1968). If the level of excitation or energising level is increased, reaction to stimuli may be more easily induced.

Stimuli that do not normally induce a response in an excitable membrane may induce an effect if the level of excitation is increased high enough.

In recent years there have been significant advances in the understanding of the structure and function of the sarcoplasmic reticulum (calcium ATPase). In contrast, almost nothing is known about the calcium release mechanism.

The calcium ATPase actively pumps calcium from the myoplasm into the inside of the sarcoplasmic reticulum. If calcium is released from the sarcoplasmic reticulum and reaches a critical threshold level, contraction takes place by a relatively well understood mechanism (Murray and Weber, 1974; Cohen, 1975).

The calcium ATPase of the sarcoplasmic reticulum keeps the concentration of calcium in the myoplasm so low that relaxation of the muscle is maintained. The mechanism of calcium release cannot be explained by a simple reversal of the pump. In fact, reversal can be induced in vitro but it is ten thousand times too slow to account for contraction in vivo. Ion channels in the SR membrane have been proposed for some time. A model with some form of activator mechanism is postulated to open the ion channels upon activation from the sarcolemma. Control of this process by an energised membrane and its phospholipid has been suggested (Lauria, 1975).

If fluidity of phospholipid can influence membrane

activation, then the drugs studied in this thesis could be acting on muscle membranes by influencing the fluidity of the phospholipid. Any defect which maintains membranes in a super-fluid or abnormally high activated state, could result in impairment of membrane function. The sarcolemma appears to be a highly dynamic fluid controlled membrane with ion channels, and the sarcoplasmic reticulum a similar type of membrane but with different transport properties and energy states associated with it. The association between these two membranes is obviously critical to normal muscle function.

In the past, theories on membrane structure have had phospholipid evenly distributed around the surface of the membrane in the form of a bilayer (Danielli and Davson, 1935). That most membranes found in biology have various regions of phospholipid bilayers is now well established (Singer and Nicolson, 1972). Recent work, using physical techniques on phospholipid model membranes and natural membranes has revealed a very dynamic organisation of difference types of phospholipid in membranes.

Membranes consist of various types of phospholipid. These different types of phospholipid in a pure state have different fluid properties. For example, artificial phosphatidylcholine membranes are more fluid than phosphatidylserine ones. Studies by Ohnishi and Ito (1974) and Ito and Ohnishi (1974) have conclusively demonstrated that in membranes with different phospholipids, specific types of phospholipid can be induced to aggregate. Thus, after aggregation there are solid and highly liquid regions or pores,

and the process is reversible. The phenomenon is known as phase-separation and has now been shown to be induced by calcium (Ohnishi et al., 1974; Papahadjopoulos and Poste, 1975).

Phase-separation in excitable membranes provides a rational explanation for the development of the action potential in nerve, ionic transport across membranes and a molecular model of calcium induced calcium release in muscle cells (Ohnishi et al., 1974). Tasaki, Watanabe and Lerman (1967) showed that external calcium was essential for developing the action potential in the squid giant axon, whereas magnesium was completely ineffective. The ionic selectivity is remarkably parallel to that for inducing phase-separations in membranes containing phosphatidylserine. Moreover, Cook, Low and Ishijima (1972) showed involvement of phosphatidylserine in nerve excitation.

Protein, phospholipid chain length and the degree of unsaturation, sterol, inorganic ions and temperature have all been shown to alter phospholipid fluidity in membranes (Chapman and Wallach, 1973; Williams and Chapman, 1971; Austin, Brown and Stewart, 1975). However, specific control mechanisms of phospholipid fluidity and the relative importance of interactions between some of these membrane components are not known. Calcium and magnesium have also been shown to reduce phospholipid fluidity or motion to differing degrees (Ohnishi et al., 1974). Thus, calcium/magnesium ratios in membranes may play an important role in controlling fluidity. The effects of these ions on specific

phospholipids are quite different. This affinity for specific phospholipids by calcium can induce phase separation within phospholipid membranes (Ito et al., 1974). The calcium-chelated ordered state and the disaggregated disordered state might be related to the two states in the theory of Tasaki (1968) on nerve excitation and the two membrane states in sarcoplasmic reticulum (Ohnishi et al., 1974; Luria, 1975).

Protein-phospholipid interaction is also thought to be important in controlling regions of phospholipid fluidity in excitable membranes (Austin et al., 1975; Brown, Bradbury, Austin and Stewart, 1975).

Energy is required to maintain the energised conformation of excitable membranes. If the supply of energy is uncoupled the membrane may be locked into the de-energised state. Cellular compensation by rapid hydrolysis of ATP may cause further depletion of energy reserves (Luria, 1975).

In summary, control of membrane fluidity has been suggested to be important in nerve and muscle membrane excitation or energy levels. Two states of lipid have been proposed, aggregated and diffuse with aggregated lipid being required for ionic flow across membranes. Halothane and most other general anaesthetics have a fluidising effect on normal membranes, increase intra-membrane bound calcium and sensitize the excitable membranes. It has been recently suggested that the sensitivity of an excitable membrane may depend on the fluidity of the phospholipids (Luria, 1975; Seeman, 1972).

Thus, an excitable membrane is more easily triggered in vitro and the resultant muscle contracture is larger after treatment with halothane, because more calcium is available for release. A higher level of intra-membrane bound calcium would increase the likelihood of calcium induced phase separation in reticulum membranes.

The postulated halothane activation of the sarcolemma membrane would predict an increase in muscle response by external triggering agents. If normal human muscle is exposed to potassium chloride (80mM) in vitro no effect occurs, but if halothane is present causes a very sharp contracture (Moulds et al., 1974a). Thus, halothane has lowered the activation energy required to activate the sarcolemma membrane. The speed of recovery from the contracture suggests that the calcium ATPase pump is not affected (Moulds et al., 1974a; Nelson and Denborough, 1975, unpublished observations). Halothane also potentiates twitch response in normal human muscle, adding further support to the hypothesis (Nelson and Denborough, 1975, unpublished observations).

Halothane may increase the activation state of the sarcoplasmic reticulum as well as the sarcolemma. Caffeine at low concentrations in frog muscle (< 1 mM) and in human muscle (< 2 mM) does not cause contracture by itself but does appear to affect the sarcolemma membrane and the excitation-contraction coupling mechanism (Bianchi and Bolton, 1967; Nelson, Austin and Denborough, 1975, unpublished observations). Low concentrations of caffeine appear to modify membrane

function by increasing the energy state of the membrane (Luttgau and Oetliker, 1968). If halothane and caffeine are combined a super-activated state is produced, calcium is released and contracture occurs. Because the activation of the membranes is an artificial one however, the recovery is slow because the membranes remain highly activated while the halothane and caffeine are present. Caffeine effects can be reversed by simply replacing the muscle buffer. Pretreatment of normal muscle with low levels of caffeine renders the samples sensitive to the same agents that malignant hyperpyrexia muscle responds abnormally to in vitro. A lower mechanical threshold in malignant hyperpyrexia muscle membranes may explain the raised calcium levels in the myoplasm following treatment by one of the various triggering agents. The proposed increase in the excitation level of malignant hyperpyrexia muscle membranes also explains the abnormal in vitro responses to potassium chloride, succinylcholine, caffeine, halothane and an increase in temperature (Moulds and Denborough, 1974c).

The mode of action of local anaesthetics on membranes is variable and depends on many factors. The charge distribution of the anaesthetic, molecular weight, pH, temperature, the amount of cholesterol, ionic strength, anaesthetic concentration, calcium concentration, the nature of membrane lipids and levels of unsaturation, can all influence the anaesthetic membrane interaction (Seeman, 1972). These same factors have been shown to affect phospholipid fluidity in membranes (Chapman et al., 1973). Studies reviewed by Seeman (1972) and Papahadjopoulos (1972) strongly

suggest that the action of local anaesthetics on membranes may be mediated via a specific interaction with acidic phospholipids.

Recent studies using Electron Spin Resonance techniques have shown that some local anaesthetics can induce significant molecular disordering and enhance the fluidity of phospholipids in natural and model membranes (Hubbell and McConnell, 1968; Hubbell, Metcalfe, Metcalfe and McConnell, 1970; Trudell, Hubbell and Cohen, 1973; Butler, Schneider and Smith, 1973; Papahadjopoulos, Jacobson, Poste and Shepherd, 1975). Very recent studies have shown that dibucaine increased the fluidity of acidic phospholipids and displaced calcium from membranes but had no effect on neutral phospholipid regions (Papahadjopoulos et al., 1975).

The apparent stimulation of calcium uptake into the sarcoplasmic reticulum may be based on the specific fluidizing effect of the local anaesthetics in regions of acidic phospholipids where ion pumps are located.

Cationic anaesthetics adsorb to the sarcoplasmic reticulum membrane preventing release of calcium and also displace sarcolemma bound calcium, blocking stimulus response coupling. Neutral anaesthetics have the opposite effect and increase the likelihood of calcium release from the reticulum. Such effects can be explained by the action of local anaesthetics on the phase-separation model of nerve excitation and calcium release from muscle membranes. Tetracaine acted antagonistically to the calcium induced phase

separations described by Ohnishi (Ohnishi et al., 1974). The structure and mode of action of tetracaine is very similar to procaine (see figure 2). Local anaesthetics block the aggregation of phosphatidylserine molecules and thereby inhibit nerve impulses or calcium release from muscle membranes.

The molecular action of dexamethasone and hydrocortisone on muscle membranes is not known. The failure of dexamethasone to affect in vitro muscle contractures in normal or malignant hyperpyrexia muscle suggests that this drug is unable to affect muscle membranes. Although hydrocortisone inhibited and relaxed caffeine + halothane contractures in normal muscle, its effect on malignant hyperpyrexia muscle is unknown.

The postulation that dantrolene is an effective inhibitor of the abnormal response of malignant hyperpyrexia muscle in vitro by normalising phospholipid fluidity and/or membrane energisation levels has some indirect evidence in support of it.

In vitro testing of skeletal muscle from patients suffering from Dystrophia Myotonica have revealed several similarities to malignant hyperpyrexia muscle. Contractures were induced by potassium chloride and succinylcholine, but no abnormal response to caffeine could be demonstrated (Moulds and Denborough, 1974d; Moulds, 1974).

Evidence of abnormal cellular membranes other than

in muscle have been demonstrated for both myopathies. Roses, Butterfield, Appel and Chestnut (1975) and Butterfield, Chestnut, Roses and Appel (1974) have presented evidence that substantiates the presence of a membrane defect in myotonic erythrocytes. There was increased membrane fluidity and decreased polarity in myotonic membranes. Diphenylhydantoin (Phenytoin) normalized fluidity in myotonic membranes but had no effect on membranes from normal patients. Diphenylhydantoin (see figure 2) has been demonstrated to be active at the membrane level and has been useful in the treatment of clinical myotonia. The implications from this study are important and the results represent one of the first properly documented membrane diseases in man attributable to phospholipid fluidity. Dantrolene is 1-{{{5- (4-nitrophenyl) -2-furanyl} methylene} amino} hydantoin (see figure 2) and has also been shown to be active at the membrane level. Its effect on phospholipid fluidity in muscle membranes is unknown and warrants further investigation. The evidence presented by Roses et al. (1975) that hydantoins affect phospholipid fluidity in human membranes suggests possible membrane interactions by dantrolene.

Temperature can be used to induce phase changes in sarcoplasmic reticulum (Inesi, Millman and Eletr, 1973). Three states of membrane lipid-protein interaction were found in the temperature region of 20-40°C. Above 20°C the lipid matrix probed by spin labels exhibited a large increase in molecular motion and a decrease in the apparent ordering of lipid alkyl chains. In addition, labels covalently bound to enzyme reative sites indicated that the motion of protein side

chains was sensitive to this transition.

Another modification, and perhaps the most important in relation to malignant hyperpyrexia, occurred between 37°C and 40°C. At this temperature the processes of calcium accumulation and ATPase activity were uncoupled. Calcium accumulation was inhibited while calcium ATPase activity and passive calcium efflux proceeded at rapid rates. This process appears to be operational in malignant hyperpyrexia membranes.

Halothane does not induce a contracture in malignant hyperpyrexia muscle in vitro at 25°C but does illicit a response at 37°C. Halothane may increase the activation state of an abnormal membrane to the point where control of fluidity and protein-lipid interaction is lost.

An increase in temperature from about 22°C to 37°C is sufficient to induce contracture in malignant hyperpyrexia muscle in vitro (Moulds and Denborough, 1974b). The increase in body temperature during an episode of malignant hyperpyrexia would compound any loss of control over membrane fluidity.

Confirmation of the presence of an abnormal fluid state in malignant hyperpyrexia muscle membranes remains for future investigation. A study on the molecular interaction of dantrolene with membranes from normal and malignant hyperpyrexia muscle may shed some light on the molecular events during the onset of the syndrome.

B: Clinical significance of the results

The results presented in this thesis suggest that dantrolene, hydrocortisone and procaine may inhibit or reverse malignant hyperpyrexia in humans.

Dexamethasone failed to produce any effect on in vitro muscle contracture. The failure of dexamethasone to affect halothane contractures in malignant hyperpyrexia muscle throws doubt on its suitability as a treatment for the syndrome. In a recent report, Isaacs showed that dexamethasone was not effective in preventing or reducing halothane-induced rigor in muscle from a susceptible patient (Isaacs, Heffron and Badenhorst, 1975). Thus, the suggested use of dexamethasone (Ellis, Clarke, Appleyard and Dinsdale, 1974) should be treated with reserve.

Pronestyl and procainamide were both less effective than procaine in antagonising muscle contracture, regardless of which in vitro model was used. The dose dependency of procaine suggests that the concentration required to produce a worthwhile effect is well above toxic levels for humans.

Over interpretation of results in this field of research is particularly noticeable. One report on the successful use of procaine in preventing malignant hyperpyrexia in swine caused considerable excitement at the time and prompted its use in humans (Harrison, 1971). However the study reported that only two out of five pigs survived. These results were not able to be repeated in different strains

of pigs (Hall, Trim and Woolf, 1972) and now some workers are suggesting the danger of its use outweighs any possible therapeutic value in humans (Hall et al., 1975).

Observations using normal muscle at high hydrocortisone concentrations imply that a very high clinical blood level of hydrocortisone is required for any effect to occur. High levels suppressed and relaxed caffeine + halothane contractures but not caffeine contractures. The relevance to treatment of malignant hyperpyrexia is therefore questionable and depends on which normal muscle model is selected as the most appropriate one. That hydrocortisone can be given in high clinical doses with little danger to the patient adds support to its use. Simultaneous use of hydrocortisone and procaine is possible. Both drugs were used in the treatment of malignant hyperpyrexia recently. The treatment was unsuccessful, however, and the patient died. The report failed to give dosage levels and drug therapy was begun very late in the development of the syndrome (Ryan and Appleyard, 1975).

The choice of dantrolene as an effective therapeutic agent in controlling malignant hyperpyrexia was confirmed by the in vitro study with muscle from a patient susceptible to malignant hyperpyrexia. The results presented in this thesis represent the first reported demonstration of the inhibiting effect of dantrolene on halothane induced muscle contracture in vitro with muscle from a susceptible patient. The fact that this drug completely suppressed halothane, potassium chloride, succinylcholine and caffeine contractures in

malignant hyperpyrexia muscle, implies that dantrolene could be an effective therapeutic agent in vivo.

A significant contribution to the search for a suitable treatment regime for malignant hyperpyrexia was the successful use of dantrolene in controlling the porcine syndrome (Harrison, 1975). Relatively high concentrations were required however, and there is some doubt about the relationship between human and porcine malignant hyperpyrexia. Drugs should not be adopted therefore until proved effective with in vitro tests at clinical concentrations.

Blood levels required to reverse the syndrome in pigs were in excess of those found in humans after oral administration. The high doses given to pigs did not induce any major side effects (Harrison, 1975). Similarly high blood concentrations of dantrolene had no effect on dogs (Ellis, Simpson, Tatham, Leighton and Williams, 1975). The in vitro study described in this report suggests that lower concentrations of dantrolene, that have been measured in humans, may be equally effective in preventing or reversing malignant hyperpyrexia.

Dantrolene appears to be relatively safe when administered to humans, particularly at the clinical concentration used during this study. Although side effects have been reported following administration of dantrolene, they have all been minor.

The main side effects found following oral

administration over a period of four weeks, with steady-state blood levels of about $6\mu\text{M}$ were general weakness, drowsiness, fatigue, dizziness, drunk feeling, headache and insomnia (Chyatte and Birdsong, 1971; Chyatte, Birdsong and Bergman, 1971).

Studies based on oral administration of dantrolene to humans imply that intravenous infusion at the concentrations suggested in this thesis, could be undertaken with safety. The speed of onset of the syndrome necessitates intravenous infusion for any drug to be effective. Intravenous administration of dantrolene has yet to be approved in the United Kingdom or the United States of America. The drug is not available to Australian doctors for administration by any route.

No drug suggested for the treatment of malignant hyperpyrexia has proved reliable. The overwhelming efficacy of dantrolene in suppressing and reversing the abnormal contractures in muscle from susceptible patients, suggests dantrolene is the most suitable drug for the treatment of malignant hyperpyrexia. The safety of dantrolene should prompt authorities to approve parenteral administration. It would appear that dantrolene is the only drug tested so far, that could increase the appalling survival rate in malignant hyperpyrexia.

APPENDIX - DATA ANALYSIS

One of the most important consequences of a study on muscle response, is the huge amount of data that is generated when compared to other methods of biochemical investigation. In the present study, eight different tests were devised to compare possible treatments for malignant hyperpyrexia. The six drugs that were tested resulted in over fifty (50) different types of experiment. Each experiment was repeated at least five times, with appropriate controls. Over eighty (80) normal subjects were selected and the biopsies performed resulted in approximately 600 muscle fibres. Each fibre studied had an average of six tension readings. Every tension reading had concentration, treatment, and positional information associated with it. Fibres were also described by patient and buffer particulars, temperature, pH, weight and length as well as other factors. The total information in the present study amounted to over thirty-five thousand variables. A comprehensive computer system was therefore developed to handle the vast amount of data generated during this study.

The computation and analysis of the data was on three distinct levels. The first level comprised the conversion of chart divisions to tension in grams, via a program in FOCAL that ran on a PDP 8/I computer. Preparing this data for analysis on a large computer, the University's UNIVAC 1108, then followed. A FORTRAN IV program assembled the data from the PDP 8/I in addition to other information

mentioned above, and then wrote the information on one of the system data files. System procedures on the UNIVAC 1108, under programmer control, were then used to add the data file to the large data file which contained information about many experiments. This up-dating procedure comprised the second level of data analysis.

The third level of analysis involved interpretation, re-organisation, and statistical procedures on the data via a programming system called P-STAT. P-STAT is a computing system for file manipulation and statistical analysis of Science data originally developed in 1962 by a team of programmers from the Princeton University computer centre (U.S.A.). P-STAT has been up-dated and revised almost annually since 1962 and is now implemented on over eight large scale computers.

A: Conversion of raw data

Fibre response was continually monitored by a Perkin-Elmer strip chart recorder. Changes in muscle tension were recorded as various peaks on the chart paper. Depending on the intensity of the response, four different settings of the amplifier attenuator and recorder would be selected as follows:-

Tension range (g)	Amplifier attenuation (arbitrary units)	Recorder (Volts full scale)
0 - 5	500	0.5
0 - 10	500	1.0
0 - 20	1000	1.0
0 - 40	2000	1.0

Settings would be changed during an experiment if the one selected before a treatment proved to be inappropriate. No contractures over 40 g were ever recorded.

The relationship of chart paper divisions to tension altered according to the range selected. A small program was written in FOCAL on a PDP 8/I mini-computer to assist in converting the approximately 70 tension readings that were associated with each biopsy (figure 20).

The program resulted in a saving of time and an increase in accuracy, particularly if two biopsies were performed in one day. The computer terminal for the PDP 8/I was located adjacent to the laboratory and connected via telephone lines to the computer which was housed in the John Curtin School of Medical Research, a distance of about one mile.

The terminal was used to input:

- 1) the experiment number,
- 2) the voltage selected on the recorder,
- 3) the sensitivity of the amplifier,
- 4) a list of chart divisions for which corresponding figures were required.

Program control was possible as data was input if a voltage or sensitivity setting had to be changed, the operator simply typed '1' and then the new settings. The program would then continue. If a mistake was made the operator typed a '99' which would automatically call an error routine. The error routine permitted any value that had been input previously to be corrected. Program control was returned to the exact position before the error was detected. Automatic print out of results would occur after every twenty-eight chart division values (one page of data). If less than 28 chart division conversions were required the operator typed '1' and then '0' in reply to the voltage setting. A full summary of the data was then printed. The summary of the data included the peak number, tension in grams, the difference between tension figures, and chart divisions to enable easy checking of the results.

B: Tension figures added to the large data file

Tension data from the summaries printed by the FOCAL program were collected, added to other information describing the experiment and physically carried to the ANU computer centre. A program, 'UPDATE' written in Fortran IV and developed on the UNIVAC 1108, was then called (figures 21, 21a). Data supplied to this program would be organised and then written to a data file. When a series of data files were collected describing a number of experiments, they were concentrated using UNIVAC 1108 system commands. The data file was saved on magnetic tape in case of system failure.

Figure 20

FOCAL program to convert chart divisions to tension (grams)

C-FOCAL, U01

```

01.05 E
01.06 A "ANU"Z,!
01.12 A "V"V;IF (V)1.12,5.05;A "S"S,!;IF (1+A(N+1))1.14,4.05
01.14 IF (1-V)1.12,2.10;S B(N)=(A(N)*.05);G 4.05

02.10 IF (S-1000)2.50,2.80;S B(N)=(A(N)*.4);G 4.05
02.50 S B(N)=(A(N)*.1);G 4.05
02.80 S B(N)=(A(N)*.2)

04.05 S N=N+1;IF (29-N)5.05,5.05
04.10 IF (A(N+I-1)+99)1.05,7.50
04.13 A A(N)
04.15 IF (A(N)+99)1.05,7.10;IF (-A(N))1.14,1.14;S N=N-1;G 1.12

05.05 T !!!!!;IF (V)1.12,5.20;S N=N-1
05.20 T "ANU"%4.02,Z,!
05.30 T "NO.      TEN      DIFF      DIV"!
05.40 FOR I=1,1,N;D 6.0
05.60 Q

06.20 T %2.0,I,"      ",%4.02,B(I),"      ",
06.22 T %4.02,B(I)-B(I-1),"      ",
06.25 T %4.02,A(I),!

07.10 A "N" I,!
07.20 T "="A(N-I);A "C?"A(N-I)
07.30 S N=N-I;G 1.12
07.50 S N=N+I-1;G 4.13
*
```

A terminal was used to input:

1. the experiment number, book and page number, date, year, muscle type, sex, temperature, buffer type and the pH of the buffer,
2. fibre number and length and weight of the fibre,
3. six comment codes which allowed a complete description of unusual experiments,
4. the peak number, concentration of a treatment, the treatment itself and the tension produced by the treatment.

All data from 1. was physically entered only once for each biopsy. The computer program however, described each fibre with this information as it was placed in the data file.

Program control was possible as data was entered. Error correction was possible by calling an error routine with a +99 for the peak number. A '-1' for the peak number would result in a question: "Finished, new fibre to be entered or totally new experiment?" A reply of 'finished' would result in the data that had been entered being written to a data file, which was then put on a system 'SAVE' tape in case of system failure.

C: Statistical analysis of the large data file via P-STAT
on the UNIVAC 1108

After processes in the preceding section were complete, a large data file containing many thousands of pieces of information was available for analysis by appropriate programs. Because of the diverse array of questions that were to be asked, a general purpose program

Figure 21 Program UPDATE

***** UPDATE *****
@HDLG,P

[illegible]

was required. The possibility of new questions arising after generation of the data necessitated a general program. The use of the P-STAT processing language was suggested by Mr. Bill Craig, Department of Clinical Science, John Curtin School of Medical Research.

P-STAT is a system of programs that does statistical operations on files of data. Version 3.06 1975, the system used to analyze data presented in this thesis, has 620 subroutines, totalling 55,000 FORTRAN source cards and 1,060 calls to various error routines.

It is a relatively easy language to use and is designed primarily for use by scientists and others who have neither the time nor the inclination to learn detailed computer programming.

The routine use of P-STAT was as follows:

1. The DATA program was called to provide an "english" version of the data file generated in section B.
2. The data in the "english" version was manually checked.
3. Mistakes found in the data file were corrected using the University of Maryland Editor on the UNIVAC 1108.
4. The DATA program was called again, this time to generate a permanent magnetic tape P-STAT version of the data.
5. P-STAT programs were written to select specific experiments that were required, and to provide standard statistical analysis of the data selected.

If more information was to be selected, a repeat

of Step 5 was all that was necessary. As data from new experiments became available, P-STAT would be used to generate a large P-STAT data file as follows:

6. Steps 1 - 4 above were repeated on the new data to be added to the large data file.
7. P-STAT was called to add the new data to the old large data file thus generating a new larger permanent P-STAT data file.

The P-STAT programs used to analyse the muscle data presented in this thesis, totalled over fifty separate programs. An example of a P-STAT program, used to select normal muscle fibres that were pre-treated with 6 μ M dantrolene before the addition of 8 mM caffeine, is shown in figure 22.

Figure 22

P-STAT program : 6 μ M dantrolene before 8 mM caffeine

```

P-STAT, VERSION 3.06, REVISION 3A, SEP 29, 75, 40K OVERLAY. (TINY VERSION)

..... P-STAT INPUT .....

1  ATTACH=HUMANMCLE $
2  NO.OP, IN = HUMANMCLE (IF CONC.1 EQ 0.0 AND TREATMENT.1 EQ 6.0, RETAIN)
3  (IF TEMP.CENT EQ 37.0, RETAIN)
4  (IF PH EQ 6.0, DELETE)
5  (IF CONC.2 EQ 2.0 AND TREATMENT.2 EQ 3.0, RETAIN)
6  (IF CONC.4 EQ 8.0 AND TREATMENT.4 EQ 1.0, RETAIN)
7  (SETX DT43 TO TENSION.4 - TENSION.3)
8  (C CONC.1-TENSION.5,BOOK.NO.,TEMP.CENT,PH,COMMENTS.1-COMMENTS.3,DT43)
9  OUT = TEMP2 $
10 PRINT=3 / TEMP2 $
11 SCAN, IN = TEMP2, DES = DES $
12 PRINT = 3 / DES $
13 PURGE = DES $
14 END $

```

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